Dynamics of biomass accumulation by *Aronia melanocarpa* cells in liquid culture

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Abstract. Cellular agriculture is an emerging field of biotechnology that aims to produce foods and other agricultural products directly from cultured cells, offering a sustainable alternative to traditional farming. In plant cell culture systems, the avoidance of herbicide application is crucial. Accordingly, we present results from a plant cell culture system optimized through adaptation with natural growth regulators. In this work, growth dynamics and consumption of key nutrients (phosphate, ammonium, and nitrate ions) by Aronia cells, grown under submerged conditions, have been investigated. The accumulation of secondary metabolites was monitored by analyzing changes in total phenolic content and antioxidant activity. The culture accumulated a maximum biomass of 7.83 ± 0.79 g/L (dry weight) on the 12th day. Our study demonstrates that by using natural growth regulators for the cultivation of Aronia cell suspension cultures, it was plausible to produce a high amount of cell biomass with increased nutritional value. The results can be used as a base point for the development of sustainable biotechnology processes for Aronia biomass production.

1 Introduction

Global agriculture is facing a major challenge to supply the growing population with food and, meanwhile, not to underestimate the ecological issues. Cellular agriculture seems to be the solution to that growing demand [1-3]. Increasing consumer nutritional awareness and ongoing technological improvements lead to the development of this industrial segment. One way to obtain food with health-promoting properties is to enrich raw materials with bioactive phytochemical compounds [4]. Plant-based active substances typically represent complex mixtures of bioactive compounds that cannot be easily substituted with synthesized analogs. The benefits of plant cell culture technologies have been widely studied for rapid production of pharmaceutically valuable plant metabolites. Nowadays, many cosmetic products contain active ingredients obtained through *in vitro* plant cell technologies [5].

*Aronia melanocarpa (Michx.) Elliott (*black chokeberry) is a shrub belonging to the Rosaceae family originating from North America [6]. It is well known for its berries rich in polyphenols and possessing remarkable antioxidant activity [7-11]. *A. melanocarpa in vitro* cultures were reported as promising producers of valuable secondary metabolites, mainly phenolic ones [12-15]. In our previous study, we demonstrated the potential of *A. melanocarpa* cell suspension culture as a prospective producer of antioxidants [16]. However, up to now there are no attempts made to introduce this culture system for commercial production of active ingredients with applications in cosmetic or food products. One of the key problems is the use of synthetic growth regulators, which are classified as herbicides and, because of their negative effects on nature and human health, are under strict regulation [18]. To overcome this issue, the application of naturally occurring growth regulators or screening of natural extracts with growth-promoting effects is considered a viable alternative [19]. However, because of the high biological activities, better stabilities, different mechanisms of action, and much lower price of synthetic growth regulators, their exchange with natural ones is a difficult task and often leads to a decrease in system productivity [19-21]. Therefore, the investigation of the main physiological characteristics of the plant in vitro cultures is the base of the next implementation of them as a technological matrix for the development of production processes for bioactive secondary metabolites [15, 16]. The aim of this study is to investigate the potential of *A. melanocarpa* cell suspension culture to grow on a medium composed of natural growth regulators and to evaluate its potential as a possible sustainable source of antioxidants with application in food and cosmetic products.

2 Materials and methods

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2.1 *Aronia melanocarpa* plant material

The cell suspension of *A. melanocarpa* was obtained as described elsewhere [16]. The cell suspension used in the current study was subcultivated every 7 days for more than 6 months on an orbital shaker (110 rpm) in 1 L flasks filled with 100 mL Woody Plant liquid medium supplemented with a 30 g/L sucrose, 1.0 mg/L 2-iP-riboside (Duchefa Biochimie, Netherlands) and 2.0 mg/L IAA (Duchefa biochimie, Netherlands), at 24°C in darkness. Inoculation was performed with 50% v/v 7-day-old suspension. Cell suspension growth was evaluated through accumulated dry biomass (ADB, g/L) and growth index (GI).

2.2 Determination of ammonia, nitrate and phosphate ions

For determination of ammonia, nitrate, and phosphate ions, culture liquids were centrifuged for 10 minutes at 4000 rpm. One milliliter of supernatant was diluted (30-fold) with distilled water and used for analyses. Nitrate, ammonium, and phosphate ions were determined by chemical test kits (Sigma Aldrich, Spectroquant® nitrate test, 1.14773.0001; ammonium test-1.4752.0001; phosphate test 1.14842.0001).

2.3 Determination of Total phenolic content

Freeze-dried biomass (0.5 g) was extracted with 70% ethanol (1:40 w/v) in an ultrasonic bath (40 kHz, 80 W) for 45 minutes and filtered through filter paper. Extracts were evaporated at 50°C under vacuum to remove the ethanol component. Resulting water fractions were collected and freeze-dried. Freeze-dried extracts (0.1 g) were dissolved in 1.0 mL methanol, then passed through a 0.45 µm PTFE filter. The samples prepared in this way were analysed for total phenolics and antioxidant activity assays.

Total phenolic content was determined by using the Folin-Ciocalteu assay as described elsewhere [16]. In brief, 180 µL of the Folin-Ciocalteu reagent was mixed with 20 µL of the investigated extract in 96-well plates. Then the plates were shaken at 37ºC for 5 minutes, and 100 µl of 7.5% sodium carbonate was added. The total reaction time was 15 min.A blank sample was prepared in the same way, but with 20 µL of methanol instead of extract. The absorbance was measured at λ = 765 nm against the blank sample (Thermo Scientific Multiskan FC Microplate Photometer equipped with SkanIt software). Total phenolic content was expressed as gallic acid equivalents per gram of dry biomass by using a standard curve (TPC, mg GAE = 4.304898 + 211.7362 x A_{765}; r^2 = 0.9969125) built with gallic acid.

2.4 Antioxidant Activity Assays

Antioxidant activities of phenolic extracts from Aronia cells were evaluated by using four in vitro methods: scavenging of DPPH radical, scavenging of ABTS radical, ferric reducing antioxidant power (FRAP), and cupric ion reducing antioxidant capacity (CUPRAC), described elsewhere [16, 25].

2.4.1 DPPH

The DPPH assay was performed by mixing 20 µL of methanol extracts of A. melanocarpa and 280 µL of 0.1 mM solution of 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) in methanol. A negative control was prepared as well by using 20 µl methanol instead of extract. The analyzed samples were transferred to a 96-well plate and then loaded to a multiplate photometer (Multiskan FC, Thermo Scientific). The plate was incubated for 15 minutes at 37ºC, and the absorbance was automatically measured at λ = 517 nm against the methanol (Thermo Scientific Multiskan FC Microplate Photometer equipped with SkanIt software). The % of DPPH radical inhibition was calculated and used to express the antioxidant activity of extracts as µM TE per gram dry weight by using a standard curve (DPPH scavenging, µM TE = 34.459944 + 6.7547127 x % Inhibition; r2 = 0.99240105), built by plotting trolox concentrations vs. % DPPH radical inhibition.

2.4.2 TEAC

The ABTS assay was performed by mixing 20 µL of methanol extracts with 280 µL ABTS radical (generated by mixing aliquots of 7.0 mM 2, 2′-azinobis (3)-ethylbenzthiazoline-6-sulfonic acid and 2.45 mM potassium persulfate for 16 h in darkness). Negative control was prepared as 20µl of methanol was added instead of the extracted sample. Prepared samples were transferred on a 96-well plate and loaded to a multiplate photometer (Multiskan FC, Thermo Scientific). The plate was incubated for 15 minutes at 37ºC, and the absorbance was measured at λ = 734 nm against methanol (Thermo Scientific Multiskan FC Microplate Photometer equipped with SkanIt software). The % of ABTS radical inhibition was calculated and used to express the antioxidant activity of extracts as µM TE per gram dry weight by using a standard curve (ABTS scavenging, µM TE = -28.335485 + 3.3667831 x % Inhibition; r^2 = 0.99428659), built by plotting trolox concentrations vs. % ABTS radical inhibition.

2.4.3 FRAP

The FRAP assay was performed by mixing 20 µL of methanol extracts with 280 µL FRAP reagent (10 parts of 300 mM sodium acetate buffer with pH 3.6, 1 part of 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM hydrochloric acid, and 1 part of 20 mM iron(III) chloride hexahydrate in water). A blank sample was prepared as 20 µl of methanol was added instead of extract. The plate was incubated for 15 minutes at 37ºC, and the absorbance was measured at λ = 593 nm against the blank sample (Thermo Scientific Multiskan FC Microplate Photometer equipped with SkanIt software). The antioxidant activity of extracts was expressed as µM

TE per gram dry weight by using standard curve (FRAP, µM TE = -0.029555531 + 450.65229 x A595; r2 = 0.99762437), built by plotting trolox concentrations vs absorption (λ = 593 nm).

2.4.4 CUPRAC

The CUPRAC assay was performed by mixing 20 µL of methanol extract with 70 µL of 10 mM copper dichloride hydrate, 70 µL of 7.5 mM neocuproine in methanol, 70 µL of 1.0 M ammonium acetate buffer (pH 7.0), and 70 µL of distilled water in a 96-well plate. A blank sample was prepared as 20 µl of methanol was added instead of extracts. The plate was incubated for 15 minutes at 37ºC, and the absorbance was measured at λ = 450 nm against the blank sample (Thermo Scientific Multiskan FC Microplate Photometer equipped with SkanIt software). The antioxidant activity of extracts was expressed as µM TE per gram dry weight by using a standard curve (CUPRAC, µM TE = -32.530419 + 2464.2078 x A450; r2 = 0.99122789), built by plotting trolox concentrations vs absorption (λ = 450 nm).

2.5. Determination of conductivity and pH

pH and conductivity were measured by using a pH/conductivity meter (InoLab,Germany; IsoLab, Germany).

2.6 Statistical Analyses

All experiments were repeated in three replicates (n = 3), and each point was analysed in three technical replicates. Data was expressed as means with standard deviations (±SD) (n = 3). The statistics and graphical presentation of the data were performed by using the software packages SigmaPlot 12 and Excel.

3 Results and discussion

3.1 Dynamics of Accumulated Dry Biomass and Growth Index of *Aronia melanocarpa* Cells in Liquid Culture.

A. melanocarpa grew intensively as a homogeneous cell suspension containing mainly single cells and small aggregates. The cell suspension reached maximum accumulation of biomass (7.83 ± 0.79 g/L) on the 12th day of cultivation. On the same day of cultivation, the growth index was 1.5±0.2 (Fig. 1).
It should be underlined that the same suspension cultivated in nutrient media supplemented with synthetic growth regulators (PIC, KIN) achieved 10.69 ± 0.79 g/L accumulated biomass on the 6th day of cultivation [16]. This could be expected because it is well known that synthetic growth regulators are more stable and can induce endogenous auxin production in plants [18-20].

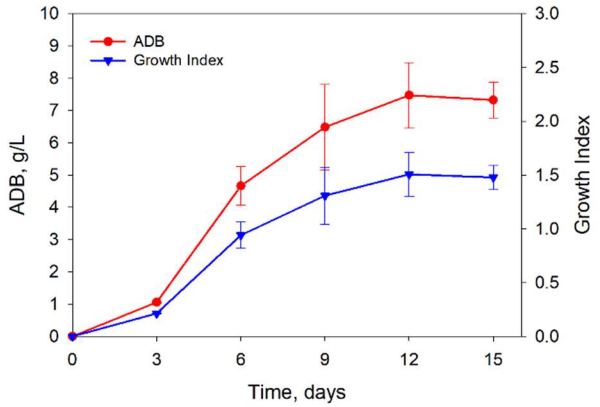

Fig. 1. Time course of growth (ADB) and changes in (GI) of cell suspension of *A.melanocarpa* cultivated under submerged conditions for 15 days.

3.2. Dynamics of ammonia, nitrate, and phosphate uptake by *Aronia melanocarpa* cells in liquid culture

The concentration of ammonium ions decreased initially from 10-12 mg/L and reached a constant concentration during the 15-day period at 1.5–3.0 mg/L (Fig. 2). At the same time concentration of nitrate ions in the culture liquid decreased smoothly to 77 mg/L on the 12th day of cultivation. This was due to the well-known mechanism of their reduction to ammonium ions and next uptake from plant cells. The time course of phosphate uptake starts from 30 mg/L, decreasing to 8 mg/L on the 12th day and a slight rising shift around 14 mg/L at the end of the growth cycle. The increase of phosphate ions in the end of the growth cycle could be due to their secretion from the cell vacuoles of plant cells dying at the end of the process. The obtained results clearly showed that inorganic basal salts in Woody Plant medium ensured the growth of *A. melanocarpa* cells in liquid culture.

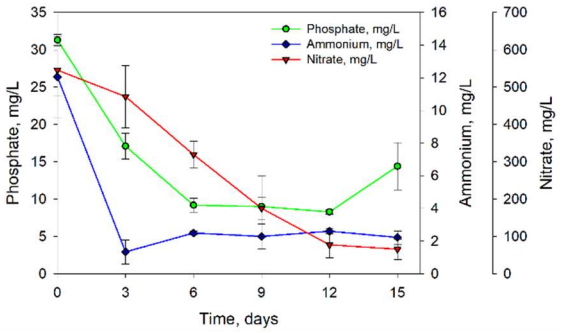

Fig. 2. Dynamics of ammonia, nitrate and phosphate ions of *A. melanocarpa* liquid cell cultures cultivated for 15 days.

3.3 Total Phenolic content

Results on biosynthesis of total phenols by *A. melanocarpa* cell suspension at submerged conditions of cultivation are presented in (Fig. 3). The time course

of total phenols biosynthesis followed biomass accumulation (Fig. 1) Maximal amounts were reached after 12 days of cultivation. (77.97 ± 10.07 mg/g dry biomass). The maximal yield of the system (1102.78 ± 121 mg/L) was also reached at this time point. Hence, we can define *A. melanocarpa* cell suspension culture as a good producer of phenolic metabolites. In a study with cells in liquid cultures, up to 660 mg/100 g DW of total phenolic acids were reported, highlighting the potential of these systems for the enhanced production of bioactive compounds [22]. Relatively high levels of phenolic compounds obtained in the study highlight the potential of *A. melanocarpa in vitro* cultures as an efficient source of phenolic metabolites. This is a good base for the next investigation of the antioxidant activities of extracts of cells of *A. melanocarpa* suspension.

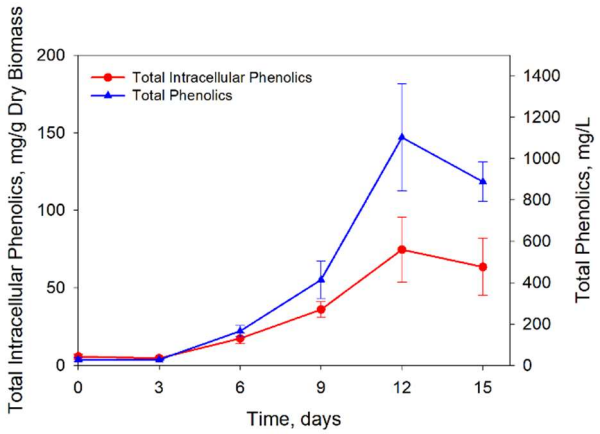

Fig. 3. Dynamics of total phenolics content and total intracellular phenolics by *A.melanocarpa* cell suspension cultures cultivated in shaking flasks for 15 days.

3.4 Antioxidant assay

The antioxidant capacity of extracts of the *A. melanocarpa* liquid cultures was assessed using four *in vitro* assays: DPPH, ABTS, FRAP, and CUPRAC. Using several methods allowed a more reliable assessment of the antioxidant potential, covering both radical-scavenging and reducing activities. Antioxidant activity (Figs. 4-7) followed the time course of phenolics accumulation (Fig. 3).

3.4.1 DPPH assay

The highest activity against DPPH (101.88±3.54 µM TE/g dry biomass) was determined on the 12th day of cultivation (Fig. 4), which corresponded to the highest amounts of total phenols accumulated in the cells of A. melanocarpa (Fig. 3). Antioxidant activity in dried fresh fruit from *A. melanocarpa* has been reported in studies of DPPH (289.5 µmol TE/100 g) [23]. However, direct comparison with the present results is not feasible due to different culture types; hence, results obtained clearly demonstrated promising radical-scavenging activity of extracts of cell suspension of *A. melanocarpa.*

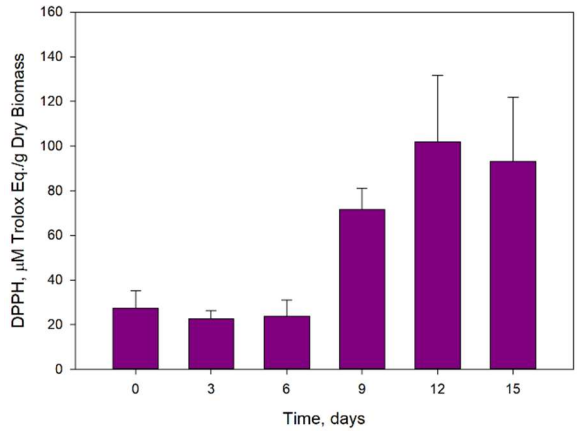

Fig. 4. DPPH determination of *A. melanocarpa* liquid cell cultures cultivated in shaking flasks for 15 days.

3.4.2 TEAC assay

Data obtained with the other radical-scavenging method, ABTS, demonstrated the same trend with a peak of maximum on the 12th day (143.46 ± 14.83 µM TE/g dry biomass) (Fig. 5). In another study, 272.85 ± 10.91 µmol TE/g of fruits from *A. melanocarpa* was reported [24]. However, the results obtained in the present study demonstrated that *in vitro* cultures from *A. melanocarpa* are a promising source for future production of antioxidant compounds.

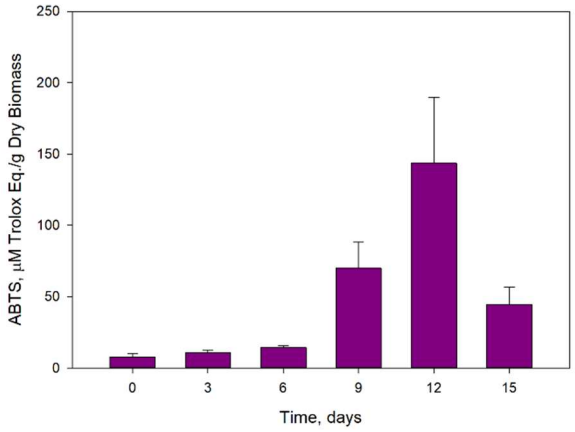

Fig. 5. ABTS determination of *A. melanocarpa* liquid cell cultures cultivated in shaking flasks for 15 days.

3.4.3 FRAP assay

Data obtained with the Ferric Reducing Antioxidant Power assay represented results also reached maximum on 12 day 244.04±20.35 µM TE/g dry biomass, while reported data from dried fresh fruits from *A. melanocarpa* 152.3 mM TE g(Fig. 6). Despite the lower ferric reducing activity of the *in vitro* culture in the present study, it possessed advantages of a controlled and standardized system allowing cultivation during the whole year.

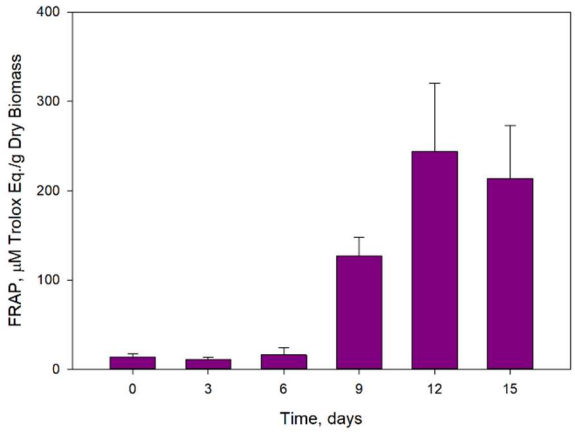

Fig. 6. FRAP determination of *A. melanocarpa* liquid cell cultures cultivated in shaking flasks for 15 days.

3.4.4 CUPRAC assay

Data obtained with the Cupric Ion Reducing Antioxidant Capacity assay showed values of 1269.13 ± 105.28 µM TE/g dry biomass on the 12th day (Fig. 7). This high value demonstrates the strong reducing capacity of the culture extracts, complementing the FRAP results and indicating that *in vitro* cultures from *A. melanocarpa* possessed a considerable amount of antioxidant compounds, such as phenolics and anthocyanins.

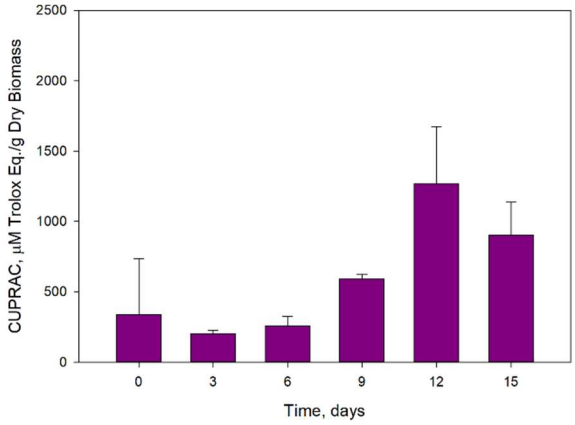

Fig. 7. CUPRAC determination of *A. melanocarpa* liquid cell cultures cultivated in shaking flasks for 15 days.

3.4 Determination of conductivity and pH

The dynamics of pH and conductivity of the culture liquid demonstrated changes throughout the cultivation period, reflecting metabolic activity of the cell suspension of *A. melanocarpa* (Fig. 8). Between the 9th and 12th day, the pH remained relatively stable (4.55) and low, indicating saturation of nutrient consumption. A slight increase on the 15th day suggests reduced metabolic activity and the beginning of nutrient depletion. Changes in conductivity are inversely proportional to biomass accumulation (Fig. 1). Conductivity continuously decreased to the 9th day (1.033–1.090 mS/cm), representing active uptake of mineral ions by the cell biomass. After the 9th day, conductivity values stabilized (around 1.100–1.300 mS/cm).

Results showed that measuring conductivity and pH is not suitable for online monitoring estimation of biomass accumulation in *A. melanocarpa* liquid cultures (Fig. 8). Both parameters showed little correlation with the actual growth dynamics due to the complex composition of secondary metabolites and changing ionic balance during culture development.

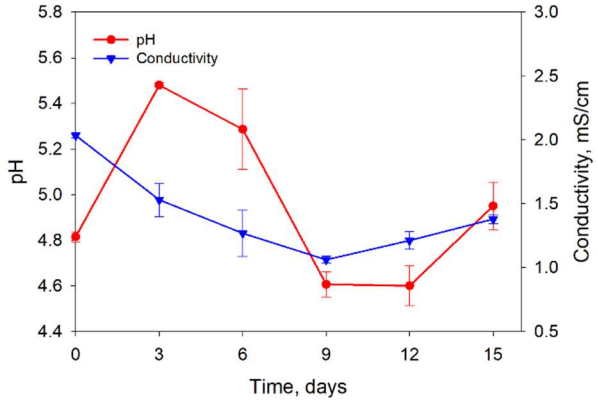

Fig. 8. Changes in pH and conductivity of *A. melanocarpa* liquid cell culture cultivated for 15 days.

4 Conclusion

A melanocarpa cell suspension culture grown in WP medium supplemented with natural growth regulators has the potential to develop secondary metabolite production processes with a promising future for application in the food and cosmetic industries, as demonstrated by the presented results. Due to the high antioxidant activity of the obtained cellular extracts and the safety of the natural growth regulators used.

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