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Development of metabolic engineering approaches to regulate the content of total phenolics, antiradical activity and organic acids in callus cultures of the highbush blueberry (*Vaccinium corymbosum* L.)

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Abstract. Blueberry (*Vaccinium corymbosum* L.) is increasingly cultivated to produce high quality berries for consumption and potential applications in medicine, nutrition and as industrial precursors. Seasonal availability sets limitations on chemical compound isolation from cultivated plants. Biotechnological solutions, such as tissue cultures and metabolic engineering, can provide sufficient amounts of plant material with reasonably high metabolite levels, which may be adjusted by different strategies. Here, we describe our approach to modifying total phenolic content (TPC), antiradical activity (ARA) and amounts of selected organic acids in *in vitro* cultures of two varieties of *V. corymbosum* by varying the growth media. TPC, ARA and acid levels were determined in mature leaves of field-grown plants and in stable callus cultures derived from leaves of varieties 'Bluecrop' and 'Duke' grown on Murashige-Skoog (MS) and Woody plant (WP) media supplemented with varying concentrations and combinations of different plant growth hormones. TPC varied from 83 mg g^{-1} dry weight (DW) to 142 mg g^{-1} DW in leaves of 'Bluecrop' and 'Duke', respectively, and correlated with their ARA with 'Duke' at the lead. For callus cultures the highest ARA, as well as the highest TPC of 94 mg g^{-1} DW was observed in 'Bluecrop' grown on WP medium with 2,4-dichlorophenoxyacetic acid (2,4-D). High level of quinic acid was found in the mature leaves of all tested varieties, while callus cultures exhibited relative increase in amounts of malic, succinic and citric acids instead. Oxalic acid was found only in callus cultures.

Key words: blueberry, *Vaccinium corymbosum*, total phenolic content, antiradical activity, organic acids, callus cultures.

INTRODUCTION

Genus *Vaccinium* comprises over 450 species of woody, perennial plants that are commonly growing in cool temperate regions of both hemispheres. Several *Vaccinium* species are cultivated to produce high value berries, while others are harvested in the wild, particularly in Northern European countries. Genus *Vaccinium* includes a few

cultivated and many wild berry species, such as blueberry (*Vaccinium corymbosum*, *V. angustifolium*), bilberry (*V. myrtillus*), cranberry (*V. macrocarpon*, *V. oxycoccus*) and lingonberry (*V. vitis-idaea*), which are recognized for their high levels of bioactive compounds (Skrovankova et al., 2015; Karppinen et al., 2016a).

Blueberry (*Vaccinium corymbosum* L.) is emerging as a high quality, nutritious berry crop plant in Northern European countries. The cultivation is increasing also in Latvia, and in 2018 reached approximately 280 ha (Karlsons & Osvalde, 2019). However, seasonal production of blueberries is not able to meet demand for consistent supply of high value bioactive compounds that have wide area of applications in nutrition, pharmaceuticals and cosmetics; therefore, biotechnological solutions are desirable. Along with solving issues of seasonal supply, they also have the potential for more sustainable production of selected compounds with less environmental impact and better control over potential contaminating factors. In addition to field crop, fruit and berry cell cultures are increasingly viewed as source of valuable ingredients for food production (Nordlund et al., 2018). Cell cultures derived from *Vaccinium* species are potentially a rich source of valuable phenolic compounds, including proanthocyanidins (Suvanto et al., 2017). Formation of callus cultures rich in anthocyanin content has also been observed in *V. corymbosum* (Ostrolucka et al., 2004). *Vaccinium* species are reported as abundant source of carotenoids, such as lutein (Karppinen et al., 2016a; Karppinen et al., 2016b), anthocyanins (Routray & Orsat, 2011) and phenolic compounds, such as, resveratrol (Rimando et al., 2004). However, industrial potential of blueberry is not met by the production levels of blueberry crop. Furthermore, industrial use requires year-round availability and uniformity only achievable through biotechnological solutions, such as blueberry *in vitro* cultures. For these solutions to become economically viable, the levels of target compounds need to be increased. Even though the berries are the most important source of bioactive compounds for food consumption, for biotechnological production also other plant tissues can be used, although much less information on bioactive compounds is available. For instance, leaves of several *Vaccinium* species were demonstrated to contain substantial amounts of the three major phenolic compounds: chlorogenic acid (polyphenol), quercetin (flavonol) and arbutin (glycosylated hydroquinone) (Stefanescu et al., 2019). Adult leaves of twenty-seven *Vaccinium* cultivars collected in three geographic regions and over three seasons of the year were shown to exhibit substantial amounts of total phenolic and flavonoid compounds and total antioxidant capacity (Páscoa et al., 2019). Higher total phenolic content (TPC) has been found in blueberry cvs. 'Duke', 'Bluecrop' and 'Nui' leaves than fruits (Fotirić Akšić et al., 2019). Flavonoids and organic acids are recognized as compounds that contribute greatly to quality and taste of blueberry fruits and have potential human health benefits. Citric, quinic, and shikimic acids have been found to be the main organic acids in fruits of different diploid and tetraploid blueberry species and cultivars (Wang et al., 2019), while another study also reported low levels of malic, succinic, and tartaric acids in cv. 'Bluecrop' (Forney et al., 2012); however, the relative amounts in different varieties and species were quite variable. Diploid and tetraploid *V. corymbosum* accessions including varieties 'Bluecrop' and 'Duke' exhibited high concentration of citric acid, but much lower concentrations of shikimic and quinic acids, compared to the other diploid species (Wang et al., 2019).

Metabolic engineering, although not a trivial task, is routinely performed in microorganisms. In plants, due to complex genomes and overlapping metabolic pathways, metabolic engineering is far from routine, although recent advances in genome editing (Zhang et al., 2019) have significantly advanced breeding for certain compounds, e.g., gamma-aminobutyric acid content in tomato (Li et al., 2018). However, heritable changes in metabolite content can only be achieved through genetic engineering and genome editing, which substantially hinders commercialization of the crops due to genetically modified organisms (GMO) regulatory issues, exemplified by the Golden Rice and Golden Rice 2 cases (Giuliano, 2017). Here, we describe our approach to modifying TPC, antiradical activity (ARA) and amount of certain organic acids in *in vitro* callus cultures of two varieties of highbush blueberry *V. corymbosum*. By varying the culture media and combinations of growth regulators, we could achieve TPC in leaf-derived calluses up to 93.89 mg g^{-1} DW. We also observed that media composition substantially affected antiradical activity and yield of organic acids. Results showed that *V. corymbosum* callus cultures may be engineered to achieve substantial amounts of high value compounds with potential health benefiting activities.

MATERIALS AND METHODS

Plant material

Commercial blueberry (*Vaccinium corymbosum* L.) cultivars (cv.) 'Bluecrop' and 'Duke' were used to derive callus cultures. Commercial cv. 'Chandler' and 'Patriot' were used in selected experiments for comparison. Leaf material of cv. 'Bluecrop', 'Chandler', 'Duke' and 'Patriot' were collected in September 2019 from field-grown plants, freeze-dried and stored in dark until extraction.

Callus cultures

V.corymbosum cv. 'Bluecrop' and 'Duke' stem cuttings and leaves were used for callus induction. Plant material was thoroughly washed with clean water and sterilized for 10 min in 15% household bleach (< 5% chlorine-based agent) solution at room temperature with constant gentle stirring followed by brief sterilization with 70% ethanol and washing with sterile water. Sterilized plant material was then placed on agarized 100% Musharige Skoog (MS) (Murashige & Skoog, 1962) or Woody plant (WP) (McCown & Lloyd, 1981) media (all from Duchefa) supplemented with 0.5 g L^{-1} 2-(*N*-morpholino)ethanesulfonic acid (MES), 30.0 g L^{-1} sucrose and 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP), 1-naphthaleneacetic acid (NAA), kinetin (KIN) and thidiazuron (TDZ) in concentrations ranging from 0.1 to 5.0 mg L^{-1} and in different combinations with the final pH 5.7 ± 0.2. Composition of growth regulators was changed to assess their potential to boost production of biologically active compounds in callus cultures. Plates were sealed with transparent parafilm and kept in versatile environmental chamber (SANYO) with photoperiod of 16 h of light (~8,000 lm) and 8 h of dark, at 25 °C, temperature on the plate surface was ~28 °C After 35 days of incubation varying degree of callus formation was observed on all tested media and plant growth hormone mixtures. Calluses were divided and transferred to fresh media every 20–30 days according to growth and quality of the callus.

Preparation of extracts for biochemical analyses

Freeze-dried and powdered plant material and callus culture biomass was extracted using 70% ethanol solution. Ratio of 1:100 (mass of freeze-dried plant /callus material : solvent volume) was used for extraction. The mixture was heated at 50 °C for 15 min followed by 18 h extraction at room temperature (22–24 °C) with continuous mixing. Dry extract weight was determined after evaporation of the solvent.

Total phenolic content assay

Total phenolic content (TPC) was determined using Folin–Ciocalteu assay adjusted for microplates with gallic acid as the standard (Singleton & Rossi, 1965; Slinkard & Singleton, 1977). Briefly, dilutions of extracts (25 μl) in 75 μl water were incubated with 25 μl 1N Folin-Ciocalteu reagent for 6 min at room temperature (22–24 °C). 100 μl 7% sodium carbonate was added to the reaction. The absorbance was measured at 760 nm using a microplate reader (TECAN Infinite 200PRO) after incubation of 90 min at room temperature in the dark. All samples were analysed in duplicates, with three technical replicates for plant leaves, and three biological replicates for calluses. Results were expressed as mg of gallic acid equivalent (GAE) per g of dry weight (DW).

DPPH Assay

Anti-radical activity was determined using the 2.2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay modified for microplates (Brand-Williams et al., 1995). Dilutions of extracts (20 μl) were mixed with 150 μM DPPH in 96% ethanol and incubated for 60 min at room temperature in the dark. Absorbance at 517 nm was measured using microplate reader (TECAN Infinite 200PRO). 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was used as the standard and the results were expressed as μM Trolox equivalent antioxidant capacity (μM TEAC). In addition, the inhibitory concentration of the extracts needed to inhibit 50% of the DPPH radicals was determined (IC50). All samples were analysed in triplicates, with three technical replicates for plant leaves, and three biological replicates for calluses; for each sample eight different dilutions were assayed.

High performance liquid chromatography – mass spectrometry (HPLC-MS) analyses of organic and phenolic acids

Methanol and acetonitrile (LC-MS grade, from Fisher Chemical), formic acid (≥ 98%, from TCI Chemicals) were used for HPLC-MS. Maleic acid (≥ 98.0%) was supplied by Supelco. Primary reference standard of chlorogenic acid and analytical standards of oxalic, succinic, malic, tartaric, citric, quinic, p-coumaric, o-coumaric, m-coumaric, shikimic and caffeic acid were purchased from Sigma-Aldrich (St. Louis, USA).

An external standard calibration was used for quantitative determination of acids in extracts of callus cultures and blueberries leaves. Stock solutions of oxalic, succinic, maleic, malic, tartaric, citric, quinic and shikimic acid standards at a concentration of 1.0 mg mL^{-1} were prepared by dissolving in water; solutions of p-coumaric, o-coumaric, m-coumaric, shikimic, caffeic and chlorogenic acid were prepared by dissolving in methanol. All stock solutions were stored at 4 °C temperature. Working solutions of mixtures of all the standards were divided into 2 parts (A and B) and were prepared immediately before analyses by diluting the stock solution with mobile phase. Seven

working solutions of part A (oxalic, succinic, maleic, malic, tartaric, citric and quinic acid) were prepared ranged from 0.05–100 µg mL^{-1} and six working solutions of part B (p-coumaric, o-coumaric, m-coumaric, shikimic, caffeic and chlorogenic acid) were prepared ranged from 0.01–50 µg mL^{-1}. The standard solution at each concentration was analysed in triplicate. All calibration curves were constructed by plotting the average peak area against concentration. Regression equations and linearity of correlation coefficient (R^2) were calculated using Microsoft Excel 2016, $p < 0.001$. Limit of detection (LOD) and limit of quantification (LOQ) were defined as values three times to noise and 10 times to noise, respectively.

HPLC-MS identification and quantification analyses of organic and phenolic acids were carried out using an Agilent 1290 Infinity series system (Agilent Technologies, Germany) coupled to an Agilent 6230 TOF LC/MS (Agilent Technologies, Germany) with electrospray ionisation (ESI). Two different methods (A and B) were used for analyses of organic and phenolic acids.

In A method, chromatographic separation of oxalic, succinic, maleic, malic, tartaric, citric and quinic acid was performed at 55 °C using a Phenomenex Rezex ROA–Organic Acid H+ (8%), 4.6×150 mm column. The elution was carried out under an isocratic elution at a flow rate of 0.3 mL min^{-1}. Mobile phase consisted of aqueous 0.1% formic acid. The injection volume was 2 µL. The mass spectrometry operating conditions were as follows: negative ionisation mode, gas temperature 285 °C, nitrogen flow rate 10 L min^{-1}, nebulizer pressure 40 psi, capillary voltage 3,500 V and applied fragmentor 75 V. The full scan mass range was set to 50–1,000 m z^{-1}. Internal reference masses 112.9856 m z^{-1} and 1033.9881 m z^{-1} (G1969-85001 ES-TOF Reference Mass Solution Kit, Agilent Technologies & Supelco) were used. Spectrum extraction and peak detection were performed with MassHunter 7.00 Software (Agilent).

In B method, chromatographic separation of p-coumaric, o-coumaric, m-coumaric, shikimic, caffeic and chlorogenic acid was performed at 30 °C using an Agilent Zorbax SB-C18, 4.6×250 mm, 5 µm column. The mobile phase consisted of aqueous 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B). The flow rate was 0.4 mL min^{-1} and gradient elution was performed according to the following program: 0 min, 0% B; 11 min, 0% B; 32 min, 40% B; 38 min, 90% B; 41 min, 90% B; 42 min, 0% B. The injection volume was 10 µL. High resolution mass spectra (HRMS) were taken on an Agilent 6230 TOF LC/MS (Agilent Technologies, Germany). The mass spectrometry operating conditions were as follows: positive ionisation mode, gas temperature 320 °C, nitrogen flow rate 12 L min^{-1}, nebulizer pressure 40 psi, capillary voltage 3,500 V and applied fragmentor 130 V. The full scan mass range was set to 100–1,000 m z^{-1}. Internal reference masses 121.0509 m z^{-1} and 922.0098 m z^{-1} (G1969-85001 ES-TOF Reference Mass Solution Kit, Agilent Technologies & Supelco) were used. Spectrum extraction and peak detection were performed with MassHunter 7.00 Software (Agilent).

Results were expressed as µg per 1 g of plant dry weight (µg g^{-1} dw).

Data analysis

The results were expressed as mean ± standard deviation (SD). Statistical analyses were done using one-way ANOVA followed by the Tukey's test. Statistical significance was defined as $P < 0.05$. Correlation between ARA and TPC was calculated as Pearson's correlation coefficient.

RESULTS AND DISCUSSION

Production of callus cultures

Biotechnological production of phytochemicals from plants requires availability of stable and uniform *in vitro* cultures, which are also optimised for production of specific compounds. *In vitro* techniques for micropropagation of *V. corymbosum* are well established, e.g., (Ostrolucka et al., 2004), however, detailed protocols for stable callus production of blueberry leaves are limited, e.g., (Dembinska-Migas et al., 1998). Here, we describe protocol for production and maintenance of callus cultures from *V. corymbosum* varieties 'Bluecrop' and 'Duke'.

Callus induction was achieved from leaves of varieties 'Bluecrop' and 'Duke' on MS media containing 2,4-D, BAP, TDZ, KIN and NAA in 0.1–5.0 mg L^{-1} concentrations. After 35 days of incubation varying degree of callus formation was observed on all tested media and plant growth regulator mixtures. Subsequently, calluses were transferred also to WP medium containing selected plant regulator mixtures and grown for 14 months. Best callus growth was observed on MS or WP media containing 0.2 mg L^{-1} 2,4-D (MS-D and WP-D), however good growth was observed also on MS media containing 1.5 mg L^{-1} 2,4-D, 2.0 mg L^{-1} BAP, 1.0 mg L^{-1} NAA and 0.1 mg L^{-1} TDZ (MS-NDBT), and 1.0 mg L^{-1} 2,4-D, 0.5 mg L^{-1} BAP, 2.0 mg L^{-1} NAA and 1.0 mg L^{-1} KIN (MS-NBKD) and 0.5 mg L^{-1} 2,4-D and 0.2 mg L^{-1} BAP (MS-DB). Mature calluses that were selected for extract preparation were pale brown to yellowish green, compact and quick to oxidize, if cut with sharp knife. For 'Bluecrop', comparable callus growth was achieved on four different variants of media (MS-D, MS-NDBT, MS-NKBT, WP-D) for 'Duke' – on two (MS-D, MS-DB).

Total phenolic content in blueberry leaves and calluses

Phenolic compounds are essential for plant growth, development and defence reactions, while their properties as natural antioxidants can have beneficial health effects in humans (Tanase et al., 2019). It is generally accepted that phenolic compounds are the substances directly responsible for the antioxidant capacity of blueberries and their positive health effects. Although most of the research concentrates on blueberry fruits, also the blueberry leaves have been shown to be a rich in polyphenols and to exhibit antioxidant capacity (Piljac-Žegarac et al., 2009). In this study total phenolic content (TPC) in the leaves of varieties 'Bluecrop', 'Chandler', 'Duke' and 'Patriot' were compared (Fig. 1, A). The lowest TPC level was found in leaves of 'Bluecrop' (83.46 ± 21.0 mg g^{-1} DW), and the highest in leaves of 'Duke' (142.10 ± 56.36 mg g^{-1} DW). Varieties 'Patriot' and 'Chandler' exhibited slightly lower TPC in leaves compared to 'Duke' (136.70 ± 47.82 mg g^{-1} DW and 119.10 ± 36.21 mg g^{-1} DW, respectively). Limited amount of information on the TPC in blueberry leaves is available, however, our results appeared to show comparable, if somewhat higher, concentrations than found in leaves of three blueberry varieties 'Bluecrop', 'Duke' and 'Nui' grown under two different management regimes in Serbia (Fotirić Akšić et al., 2019).

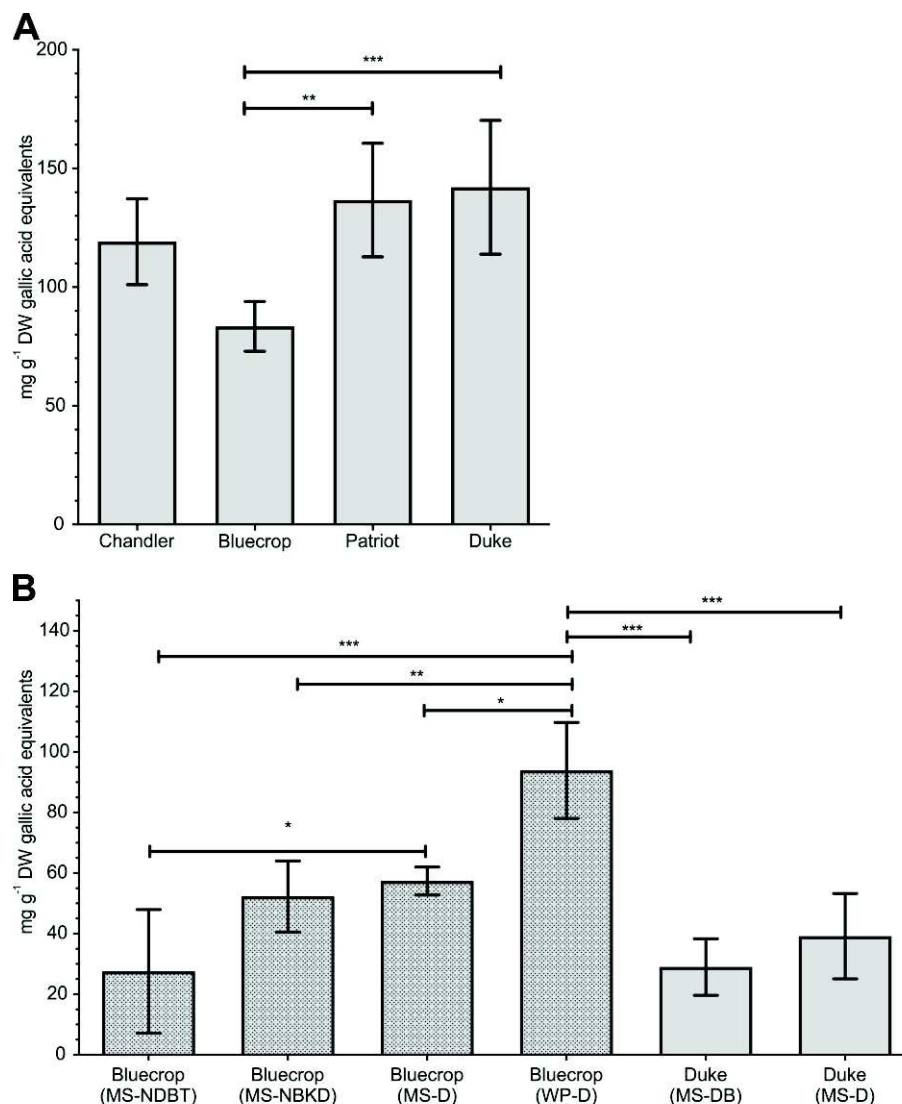

Figure 1. Total phenolic content in leaves of *V. corymbosum* varieties (A) and calluses grown on different cultivation media (B).

Results are expressed as mean ± SD mg g^{-1} DW, n = 3, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ (ANOVA). MS-NDBT – MS media with 1.5 mg L^{-1} 2,4-D, 2.0 mg L^{-1} BAP, 1.0 mg L^{-1} NAA and 0.1 mg L^{-1} TDZ; MS-NBKD – MS media with 1.0 mg L^{-1} 2,4-D, 0.5 mg L^{-1} BAP, 2.0 mg L^{-1} NAA and 1.0 mg L^{-1} KIN; MS-D – MS media with 0.2 mg L^{-1} 2,4-D; WP-D – WP media with 0.2 mg L^{-1} 2,4-D; MS-DB – MS media with 0.5 mg L^{-1} 2,4-D and 0.2 mg L^{-1} BAP.

In contrast to the TPC in leaves, the highest concentrations in callus cultures were achieved in calluses induced from 'Bluecrop' leaves, while calluses from 'Duke' exhibited lower TPC (Fig. 1, B). Modifications in culture media showed clear effect on the TPC. In the case of both varieties, addition of the 2,4-D alone to MS media increased production of phenolic compounds compared to combinations of 2,4-D with the other hormones. The highest TPC was observed in 'Bluecrop' callus cultures grown on WP

media supplemented with 2,4-D (93.89 ± 15.86 mg g^{-1} DW). There is a lack of information on specific effects of 2,4-D in plants from genus *Vaccinium*. However, effects of this plant growth regulator have been studied in other species. 2,4-D modulated gene expression in auxin, ethylene and abscisic acid pathways, as well as regulated a wide variety of other cellular functions in *Arabidopsis* based on an Affymetrix microarray study (Raghavan et al., 2005). Increase of antioxidant production and expression of antioxidative enzymes in response to 2,4-D has been shown in peas (Pazmiño et al., 2011). Regulatory effects on flavonoid biosynthesis has been shown in wheat (Pasquer et al., 2006). In callus cultures, 2,4-D showed concentration dependent effect on production of phenolic compounds. In *Moringa oleifera* callus cultures yields of phenolic compounds decreased with increasing 2,4-D concentrations, whereas combination of 2,4-D and kinetin showed positive correlation (Hamany Djande et al., 2019). Our data shows that 2,4-D alone at low concentration (0.2 mg L^{-1}) boosts production of phenolic compounds and increases antioxidative capacity, whereas when used at higher concentration (1.5 mg L^{-1}) in combination with other growth regulators, phenolic compound yields are lower. Observed effects might be due to induction of specific biosynthesis and defence pathways; however, additional gene expression and metabolic profiling analyses are needed to further substantiate this observation. Overall, our results emphasise both the importance of micronutrient and macronutrient content in the MS and WP media for induction of plant secondary metabolite production, and the choice of plant growth regulators and their concentrations.

Antiradical activity in blueberry leaves and calluses

Table 1. Antiradical activity of *V. corymbosum* leaf and callus extracts. Activity is expressed as mean±SD of IC50 μg ml^{-1} extract and μM Trolox g^{-1} DW, n = 3

Sample	IC50 (μg mL^{-1}) ± SD	TEAC (μM Trolox g^{-1} DW) ± SD
Leaves		
'Chandler'	218.40 ± 7.56	705.16 ± 112.5 *
'Bluecrop'	439.04 ± 18,13	455.73 ± 86.08
'Patriot'	228.63 ± 56.49	1066.01 ± 180.46 *+
'Duke'	141.66 ± 43.64	1302.90 ± 284.81 *+
Callus cultures		
'Bluecrop' (MS-NDBT)	856.44 ± 175.30	83.59 ± 61.75
'Bluecrop' (MS-NBKD)	454.27 ± 34.88	340.48 ± 95.52
'Bluecrop' (MS-D)	456.97 ± 121.77	296.52 ± 223.75
'Bluecrop' (WP-D)	178.82 ± 69.41	750.86 ± 196.30 ¶
'Duke' (MS-DB)	1176.44 ± 674.47	123.14 ± 37.76
'Duke' (MD-D)	1028.22 ± 215.48	164.68 ± 4.49

* Statistically significantly higher TEAC compared to 'Bluecrop' ($p < 0.01$); + Statistically significantly higher TEAC compared to 'Chandler' ($p < 0.01$); ¶ Statistically significantly higher TEAC compared to 'Bluecrop' (MS-NDBT) ($p < 0.001$); 'Bluecrop' (MS-NBKD) ($p < 0.05$); 'Bluecrop' (MS-D) ($p < 0.01$); 'Duke' (MS-DB) ($p < 0.001$); 'Duke' (MD-D) ($p < 0.001$).

Antiradical activity of leaf and callus extracts was expressed as μM Trolox equivalent antioxidant activity (TEAC) and IC50 of each extract were calculated. Results show that antiradical activity strongly correlated with TPC (Pearson's correlation

coefficient $r = 0.966$, $p < 0.0001$). Leaves of 'Bluecrop' showed the lowest activity among leaf samples, while leaves of 'Duke' exhibited the highest antiradical activity (Table 1). In calluses low antiradical activity was observed for both variants of calluses produced from 'Duke'. In the case of 'Bluecrop', the highest activity was observed in calluses grown on WP-D media (750.86 ± 196.30 μM Trolox g^{-1} DW), while the lowest in MS-NDBT calluses (83.59 ± 61.75 μM Trolox g^{-1} DW) (Table 1). IC50 values are shown in Table 1. In general, correlation between TPC and antiradical activity was observed, similarly to the study with *V. corymbosum* and *V. myrtillus* in Italy (Giovanelli & Buratti, 2009).

Content of organic and phenolic acids in blueberry leaves and calluses

Organic acids in berries play an important role in determining the organoleptic properties of fruits; however, these phytochemicals are also useful as antioxidants, preservatives, acidulants, and drug absorption modifiers in pharmaceutical and other industries (Mikulic-Petkovsek et al., 2012). Organic acids in leaves of blueberry varieties 'Bluecrop', 'Chandler', 'Duke' and 'Patriot', and calluses obtained from varieties 'Bluecrop' and 'Duke' were determined with two HPLC-MS methods described in the Methods section.

Differences in organic and phenolic acid content between leaves and callus cultures were observed (Table 2). Tartaric acid was detected only in leaf extracts and in 'Bluecrop' calluses grown on MS-NBKD media. Oxalic acid was found only in calluses. Concentrations of maleic acid, shikimic, chlorogenic and caffeic acids were higher in leaves. Both calluses and leaves contained high concentrations of citric acid, however in 'Duke' calluses concentration was lower compared to leaves. In 'Bluecrop' calluses citric acid concentration varied depending on the media used for cultivation with the lowest yield in MS-NDBT calluses (6,488 ± 317 μg g^{-1} DW) and the highest in MD-D calluses (13,696 ± 155 μg g^{-1} DW). Calluses contained more malic acid than leaves, except for 'Bluecrop' grown on WP-D media. Higher yields of succinic acid were also achieved in calluses. Chlorogenic acid was the dominant phenolic acid in leaves, while its concentration in calluses was significantly lower and varied depending on the media composition. Cultivation on MS-D media allowed to reach higher chlorogenic acid content in 'Bluecrop' calluses, but this effect was not observed in 'Duke' calluses. The highest production of chlorogenic acid was observed in 'Bluecrop' calluses grown on MS-NBKD media. Caffeic acid concentrations widely varied amongst leaves of different varieties, but all were significantly higher than in calluses. There are few studies that have reported the effects of growth regulators on production of caffeic acid derivatives in *in vitro* cultures. In *M. oleifera* callus cultures, 2,4-D upregulated production of caffeic acid derivatives, with some derivatives being present only in calluses grown on 2,4-D (Hamany Djande et al., 2019). In our study no significant differences in caffeic acid production were detected between *V. corymbosum* calluses grown in presence of different growth regulators and their combinations. Additional phenolic compound analyses and quantification are needed to elucidate effects of media and growth regulators on specific secondary metabolites.

Table 2. Content of organic and phenolic acids in blueberry leaves and calluses. Data expressed as mean ±SD (n = 3), LOD – limit of detection, LOQ – limit of quantification. Values with the same letter within a column are not significantly different at $P < 0.0$

	Malic acid	Chlorogenic acid	Quinic acid	Citric acid	Oxalic acid	Succinic acid	Maleic acid	Tartaric acid	Shikimic acid	Caffeic acid	o-coumaric acid	p-coumaric acid	m-coumaric acid
‘Chandler’	12,233 ± 71^{a}	39,282 ± 223^{a}	31,976 ± 363^{a}	7,578 ± 69^{a}	< LOD	1,121 ± 49^{a}	1,080 ± 85^{a}	600 ± 38^{a}	318 ± 8^{a}	325 ± 30^{a}	24.4 ± 7.8^{a}	16.4 ± 5.6^{a}	17.3 ± 2.8^{a}
‘Duke’	9,873 ± 62^{b}	39,261 ± 526^{a}	28,645 ± 237^{b}	9,425 ± 67^{b}	< LOD	694 ± 28^{b}	608 ± 38^{b}	180 ± 21^{b}	288 ± 6^{b}	32 ± 9^{bc}	1.3 ± 0.4^{b}	0.4 ± 0.2^{b}	0.4 ± 0.2^{b}
‘Patriot’	11,281 ± 198^{c}	49,264 ± 834^{b}	34,253 ± 572^{c}	5,474 ± 144^{c}	< LOD	915 ± 21^{c}	737 ± 55^{c}	349 ± 19^{c}	417 ± 4^{c}	61 ± 2^{c}	5.3 ± 2.4^{b}	5.0 ± 0.8^{b}	3.7 ± 1.5^{c}
‘Bluecrop’	11,521 ± 225^{ac}	31,303 ± 372^{c}	32,290 ± 490^{a}	7,720 ± 119^{a}	< LOD	646 ± 23^{b}	407 ± 31^{d}	176 ± 32^{b}	242 ± 3^{d}	147.3 ± 1.4^{d}	1.1 ± 0.3^{b}	4.7 ± 0.3^{b}	0.36 ± 0.12^{b}
Duke (MS-DB)	22,313 ± 662^{d}	17.3 ± 0.6^{d}	99 ± 25^{d}	5,902 ± 107^{c}	6016 ± 181^{a}	855 ± 91^{bc}	39 ± 3^{e}	< LOD	< LOQ	9.2 ± 1.3^{b}	0.9 ± 0.3^{b}	0.6 ± 0.1^{b}	0.72 ± 0.11^{b}
Duke (MS-D)	19,385 ± 47^{e}	25 ± 3^{d}	485 ± 15^{d}	5,129 ± 82^{c}	5287 ± 67^{b}	848 ± 21^{bc}	59 ± 5^{e}	< LOD	4.2 ± 1.0^{e}	11 ± 3^{b}	0.80 ± 0.07^{b}	0.3 ± 0.1^{b}	0.7 ± 0.2^{b}
Bluecrop (MS-NDBT)	13,554 ± 342^{f}	40 ± 3^{d}	< LOD	6,488 ± 317^{d}	5467 ± 151^{b}	2,225 ± 6^{d}	74.9 ± 1.2^{e}	< LOD	16.9 ± 0.6^{f}	14 ± 2^{b}	1.1 ± 0.2^{b}	3.1 ± 0.6^{b}	1.0 ± 0.2^{bc}
Bluecrop (MS-NBKD)	24,503 ± 459^{g}	467 ± 33^{d}	396 ± 95^{d}	7,970 ± 99^{a}	4070 ± 191^{c}	1,425 ± 34^{e}	368 ± 30^{d}	62.0 ± 0.3^{d}	17.3 ± 0.8^{f}	17 ± 3^{b}	1.68 ± 0.10^{b}	2.9 ± 0.4^{b}	0.39 ± 0.13^{b}
Bluecrop (MS-D)	23,836 ± 281^{g}	168 ± 60^{d}	922 ± 45^{d}	13,696 ± 155^{e}	4299 ± 314^{c}	1,258 ± 166^{a}	338 ± 8^{d}	< LOD	25.8 ± 0.8^{f}	8 ± 3^{b}	2.5 ± 0.3^{b}	1.5 ± 0.5^{b}	0.07 ± 0.03^{b}
Bluecrop (WP-D)	7,972 ± 37^{h}	86 ± 12^{d}	900 ± 8^{d}	8,702 ± 196^{f}	1752 ± 73^{d}	764 ± 28^{b}	203 ± 22^{f}	< LOD	7.89 ± 0.10^{ef}	7.2 ± 0.2^{b}	1.43 ± 0.05^{b}	3.2 ± 0.2^{b}	0.20 ± 0.07^{b}

Biosynthesis and accumulation of organic acids in various *Vaccinium* species have been described before (Laaksonen et al., 2010; Phillips et al., 2010; Forney et al., 2012), but there have not been any reports about modulation of organic acid composition in calluses. Mikulic-Petkovsek et al. (2012) reported that tartaric acid was not detected, and malic acid was found at low levels in *V. corymbosum* berries. Another study reported tartaric acid in *V. corymbosum* 'Bluecrop' and 'Patriot' berries (Çelik et al., 2012). In this study we showed very high level of malic acid in both leaves and calluses and comparatively high levels of tartaric acid in leaves. In general, our results clearly indicated that production of organic acids in *V. corymbosum* calluses can be boosted by modifications in cultivation media. In this study, increase in citric, succinic, malic and oxalic acids was achieved. In addition, even though the highest total phenolic content and antiradical activity was reached in 'Bluecrop' calluses grown on WP-D media, low concentrations of chlorogenic and caffeic acids were found in these samples. Since these compounds are generally implicated in the high antiradical activity of plant extracts, this finding indicates that other phenolic compounds might be responsible for the observed high antioxidative capacity in our study.

CONCLUSIONS

Successful establishment of callus cultures from *V. corymbosum* serve as a precondition for further metabolic engineering to boost production of valuable biologically active compounds. Modifications of micronutrient, macronutrient and plant growth factor composition and concentrations significantly affect production of organic and phenolic acids in callus cultures.

Total phenolic content clearly correlates with antiradical activity. Further in-depth chemical composition analyses are necessary to identify the phenolic compounds that are responsible for this activity.

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