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Metabolism of glutathione and ascorbate in lingonberry cultivars during *in vitro* and *ex vitro* propagation

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Abstract

Lingonberry (*Vaccinium vitis-idaea* L. ssp. *vitis-idaea* Britton) cultivars Regal, Splendor, and Erntedank were obtained by conventional softwood cuttings (taken as a control), by *in vitro* shoot proliferation of node explants, and by adventitious shoot regeneration from excised leaves of micropropagated shoots. In the plants propagated *in vitro*, the total ascorbate content increased and its pool was more oxidized, the total glutathione content also increased but its pool became more reduced. The leaves of plants obtained from the *in vitro* culture showed significantly higher antioxidant enzyme activities except for dehydroascorbate reductase which was at a similar level in all plants. Total soluble phenolics, tannins, and flavonoids were enhanced in fruits of *in vitro*-propagated plants whereas in leaves, the levels of these metabolites (except flavonoids) were higher in *ex vitro* derived plants. The total radical scavenging capacity was enhanced in berries of the *in vitro* propagated plants. It is suggested that the active morphogenetic process, characterized by intensive formation and scavenging reactive oxygen species is reflected in the activities of antioxidant enzymes and metabolites. The reduction potential of glutathione is the most important parameter which determines patterns of growth and differentiation in the investigated plants.

Additional key words: antioxidants, ascorbate-glutathione cycle, flavonoids, phenolics, reduction potential, *Vaccinium vitis-idaea*.

Introduction

Lingonberry (*Vaccinium vitis-idaea* L.) is a commercially important fruit crop with a great medicinal value pertaining to its high antioxidant properties (Jaakola *et al.* 2001, Wang *et al.* 2005, Lätti *et al.* 2011). Lingonberry plants are rich sources of antioxidants, especially phenolic compounds, such as anthocyanins, flavonoids, and tannins. Reports have shown that lingonberry exhibit anticancer activity and that their extracts can potentially induce apoptosis of human leukemia HL-60 cells (Wang *et al.* 2005). Lingonberry plants are heterozygous, so they are normally propagated by vegetative methods to achieve genetically identical offspring and to preserve advantageous characteristics. The conventional vegetative propagation method is a softwood cutting. The tissue culture technique is a more advanced method of micropropagation offering rapid production and numerous clones of plants from the single mother plant. The tissue culture of lingonberry plants can be obtained either from node sections or from leaves (Debnath 2011).

The enzymes of the ascorbate-glutathione cycle (also known as Halliwell-Asada pathway) play a key role in the antioxidant metabolism, especially in leaves. This cycle involves ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase. The intermediate metabolites in the cycle are ascorbate, dehydroascorbate, monodehydroascorbate, and glutathione (Noctor and Foyer 1998). The cycle efficiently scavenges hydrogen peroxide (H_2O_2), especially in chloroplast, preventing oxidative damage and maintaining redox level of the cell. A link between

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Abbreviations: AFR - ascorbate free radical; APX - ascorbate peroxidase; AsA - ascorbic acid; CE - catechin equivalent; DHA - dehydroascorbate; DHAR - dehydroascorbate reductase; DPPH - 2,2-diphenyl-1-picrylhydrazyl; GAE - gallic acid equivalent; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; LC - plants propagated from leaf cultures; MDA - malondialdehyde; MDHAR - monodehydroascorbate reductase; NC - plants propagated from node cultures; SC - softwood cutting-derived plants.
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ascorbate metabolism and accumulation of phenolic compounds has been shown (Thomas *et al.* 1992) and may be connected, in particular, with the role of ascorbate in metabolism of phenols in apoplast and in scavenging phenoxyradicals (Horemans *et al.* 2000). The dependence of ascorbate metabolism, catalase activity, and accumulation of H_2O_2 on cell differentiation and growth stage has been shown (Verma *et al.* 2008), in particular, it was demonstrated that calli are more tolerant than differentiated tissues to oxidative stress (Shekhawat *et al.* 2010).

The propagation method influences growth habit of plants (Debnath 2011, Saez *et al.* 2012). Significant morphological differences have been observed in plants obtained from softwood cuttings and tissue culture. It has been shown that tissue culture-derived lingonberry plants are superior over stem cuttings in terms of number of stems, branches, leaves, and rhizomes but produced less vigorous shoots and smaller berries (Debnath 2006). Similarly, some differences in morphology have been observed in plants derived from the *in vitro* micropropagation using node tissues and leaf tissue culture (Debnath 2005a).

The aim of the present study was to compare antioxidant enzymes, reduced and oxidized ascorbate and glutathione, soluble phenolic content, flavonoids, anthocyanin, tannin, and total radical scavenging capacity in three contrasting lingonberry cultivars propagated by three different methods to determine possible involvement of reduction levels of ascorbate and glutathione and of reactive oxygen species in morphogenetic processes in plants.

Materials and methods

Lingonberry (*Vaccinium vitis-idaea* L. ssp. *vitis-idaea* Britton) cultivars Regal, Splendor, and Erntedank were propagated by three different methods: *1*) by softwood cutting which is the most commonly used method of vegetative propagation employed for growing commercially important plants (SC); *2*) by micropropagation of nodal explants obtained from mother plant (NC); and *3*) by regeneration from leaves excised from micropropagated shoots (LC) (Debnath 2005b,c). Culture conditions were described in detail by Debnath and McRae (2002). The morphological data including plant height, number of rhizomes per plant, number of branches per rhizome, number of branches per plant, leaves per branch and leaves per plant, berry mass, and berry diameter were collected from 15 plants per treatment. Fresh young leaves and mature ripe fruits from 15 plants per treatment were harvested for biochemical assays and immediately frozen in liquid nitrogen and transferred to -80 °C until extraction. All the experiments were done in triplicate.

Chlorophyll (Chl) *a* and *b* content was measured according to Arnon (1949). For determination of reduced and oxidized ascorbate and glutathione, leaves were ground to powder in a mortar and a pestle with liquid nitrogen and homogenized with 2 % (m/v) metaphosphoric acid. Homogenate was centrifuged at 2 100 g and 4 ºC for 20 min. Supernatant was used for measurement of reduced and oxidized ascorbate and glutathione. Ascorbate (AsA) and dehydroascorbate (DHA) were determined according to Kampfenkel *et al.* (1995), the absorbance was recorded at 525 nm using *Biochrom Ultrospec 4300* spectrophotometer (Amersham, UK). Reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined according to Zaharieva and Abadía (2003). The method is based on the reaction of 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) with GSH forming 5-thionitrobenzoic acid (TNB) that absorbs at 412 nm. The oxidized glutathione (GSSG) was measured after being reduced by glutathione reductase.

Leaves were ground to powder and homogenized on ice in 1 cm^3 of 50 mM MES/KOH buffer (pH 6.6) containing 40 mM KCl, 2 mM $CaCl_2$, and 1 mM sodium ascorbate. The homogenate was centrifuged at 12 000 *g* and 4 °C for 10 min. The activities of enzymes of the ascorbate-glutathione cycle were measured according to Murshed *et al.* (2008) with modifications. The assay medium for ascorbate peroxidase (APX, EC 1.11.1.11) was 50 mM potassium phosphate buffer (pH 7.0) containing 0.25 mM sodium ascorbate and the sample extract. The reaction was started by adding H_2O_2 (final concentration 2.5 mM) and the decrease in reaction rate was determined spectrophotometrically by absorbance change at 290 nm (coefficient of absorbance, $\varepsilon = 2.8$ mM^{-1} cm^{-1}). Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was measured at 265 nm ($\varepsilon = 14$ mM^{-1} cm^{-1}). The assay buffer contained 50 mM HEPES buffer (pH 7.0), 0.1 mM EDTA, 2.5 mM GSH, and the leaf extract. The reaction was initiated by adding freshly prepared DHA (final concentration of 0.8 mM). Monodehydroascorbate reductase (MHAR, EC 1.6.5.4) activity was measured in 50 mM HEPES buffer (pH 7.6) containing 2.5 mM AsA, 0.25 mM NADH, and the extract. The assay was initiated by adding 0.4 U cm^{-3} of ascorbate oxidase and the reaction rate was monitored at 340 nm ($\varepsilon = 6.22$ mM^{-1} cm^{-1}). Glutathione reductase (GR, EC 1.8.1.7) activity was measured in 50 mM HEPES buffer (pH 8.0) containing 0.5 mM EDTA, 0.25 mM NADPH, and the leaf extract. The reaction was started by adding GSSG to final concentration of 1 mM. Catalase (CAT, EC 1.11.1.6) activity was measured at 240 nm according to Aebi (1974).

Soluble phenolics and other compounds were extracted from fruits and leaves in 80 % (v/v) acetone with 0.2 % (m/v) formic acid in the ratio of 1:10 with 8-h shaking at 4 ºC which was found to be the best extraction solvents among ethanol, methanol, and acetonitrile at various aqueous mixtures with different shaking periods. Homogenous mixture of samples and solvent was then

centrifuged at 20 000 g for 20 min. The residue was extracted twice under the same conditions and the supernatants were mixed together and further diluted to make the working concentration of 25 g dm^{-3} for fruits and 1 g dm^{-3} for leaves.

Total soluble phenolic content in both leaves and fruits was determined using Folin-Ciocalteu reagent as described by Chandrasekara and Shahidi (2011) with modifications. The Folin-Ciocalteu reagent (0.5 cm^3) was added to centrifuge tubes containing 0.5 cm^3 of extracts and mixed well. Saturated sodium carbonate solution (1 cm^3) was added to each tube to neutralize the reaction. The final volume was adjusted to 10 cm^3 by water and vortexed for 1 min. The tubes were kept in dark at room temperature for 35 min and then centrifuged at 4 000 *g* for 10 min. The absorbance was measured at 725 nm. Total soluble phenolic content of each sample was determined using the gallic acid standard curve and expressed as gallic acid equivalents (GAE) per berry or leaf fresh masses.

Total anthocyanin content was measured according to Foley and Debnath (2007). The method is based on reversible conversion of anthocyanins from their oxonium form to hemiketal form. Absorptions at 510 and 700 nm were measured in buffers at pH 1.0 and 4.5 and the difference between the two values was used to determine total anthocyanin content. Total flavonoid content was measured by aluminum chloride colorimetric assay (Zhishen *et al.* 1999). The extract (1 cm^3) or standard solution of catechin (0.5 mg cm^{-3}) was mixed with 4 cm^3 of water followed by addition of 0.3 cm^3 of 5 % (m/v) $NaNO_2$, 0.3 cm^3 of 10 % (m/v) $AlCl_3$ (after 5 min) and 2 cm^3 of 1 M NaOH (1 min later), the volume was adjusted to 10 cm^3 (with water). The absorbance was measured at 510 nm. Total flavonoid content was expressed as catechin equivalent (CE) per leaf or fruit fresh masses.

Tannin (proanthocyanidin) content was determined by the method developed by Chandrasekara and Shahidi (2011). The 0.5 % (m/v) vanillin-HCl reagent (5 cm^3) was added to 1 cm^3 of the extract, mixed thoroughly and incubated at room temperature for 20 min. A separate blank for each sample was read with 4 % (v/v) HCl in methanol. The absorbance was read at 500 nm and the content of proanthocyanidins was expressed as CE per leaf or fruit fresh masses.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was conducted according to the method of Brand-Williams *et al.* (1995) with modifications. The stock solution of 1 mM DPPH in methanol was diluted to 60 μM, 1.9 cm^3 of the latter was mixed with 0.1 cm^3 of fruit or leaf extracts, shaken vigorously, and left in dark for 20 min. The absorbance was read at 515 nm. The scavenging capacity was expressed as percentage of inhibition of DPPH consumption. The gallic acid standard curve was used to express the results as GE equivalent.

For analysis of phenolic compounds by high-performance liquid chromatography (HPLC), diluted supernatants of berries were evaporated at room temperature for 2 to 4 d in dark and lyophilized at -50 °C for 72 h. Freeze dried samples (10 g) were extracted in aqueous methanol solution (1 dm^3) and filtered through 0.45 μm polytetrafluoroethylene membrane syringe filter. The reversed phase HPLC analysis was carried out using an *Agilent 1100 LC/MSD* trap system (*Agilent Technologies*, Palo Alto, CA, USA). A C18 column (4.6 × 150 mm) with 5 μm particle size (*Chromatographic Specialities*, Brockville, ON, Canada) was used. The eluents were 0.5 % (v/v) aqueous formic acid (A) and acetonitrile:methanol (95:5) (B) with initial gradient of 85 % solvent A at 0 min to 0 % solvent A and 100 % solvent B at 30 min. Flow rate was 1.0 cm^3 min^{-1} and injection volume was 0.09 cm^3. Compounds of interest were detected using UV-spectra and retention times. Mass spectra were used for confirmation of identity of compounds using a liquid chromatography/ mass selective detector (LC/MSD) ion trap system in electron spray ionization (ESI) negative ion mode. Authentic standards were used for identification and making calibration curves for quantification. HPLC was run in MS/MS mode for identification of sugar units attached to phenolics.

All the experiments were repeated at least three times. Data in the text, tables, and figures are expressed as means ± SD of three replicates (15 plants per each treatment). Data for all characteristics were subjected to *ANOVA* using the SAS statistical software package (*Release 8.2*; SAS Institute, Inc., Cary, NC, USA). *F*-tests were evaluated at $P \leq 0.05$. Differences among treatments were further analysed using Duncan's multiple range test.

Results

In all cultivars, plants obtained by *in vitro* culture (NC and LC) were superior to *ex vitro* SC plants in terms of number of shoots, branching, and rhizome, whereas LC plants were characterized by higher number of leaves per branch but less secondary branching as compared to NC plants (Figs. 1 and 2). Fig. 2 shows different morphological characteristics in three investigated cultivars obtained by three methods of propagation. The data show variability both in relation to the cultivar and to the method of propagation. Notably, many vegetative characteristics (such as height, number of rhizomes per plant, and leaves per branch) increased in the tissue propagated plants (more in LC than in NC) as compared to the SC plants. On the contrary, the number of berries per plant, the average mass of berry, and berry diameter were lower in the tissue culture plants compared to those in the SC plants being the lowest in the NC plants. The same tendency was observed in the content of Chl *a* and *b* which was lower in the tissue culture propagated plants than in the SC plants (Fig. 2).

The content of total ascorbate (AsA + DHA) and total glutathione (GSH + GSSG) differed both depending on the cultivar and on the method of propagation (Fig. 3). The total ascorbate content was approximately the same in all three SC and NC cultivars but higher in the leaf tissue of LC. This increase was the least in Regal, more in Splendor, and more than 30 % in Erntedank. Among SC, the content of DHA was highest in Splendor. DHA content increased significantly in all LC cultivars as compared to SC but less in NC. The content of total

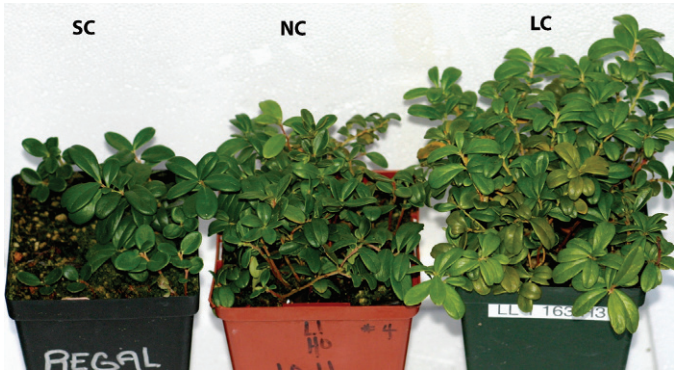

Fig. 1. Lingonberry cultivar Regal propagated by different methods. SC - stem cutting-derived plant (control); NC - node culture-derived plant; LC- leaf culture-derived plant. Plant age - 4 years.

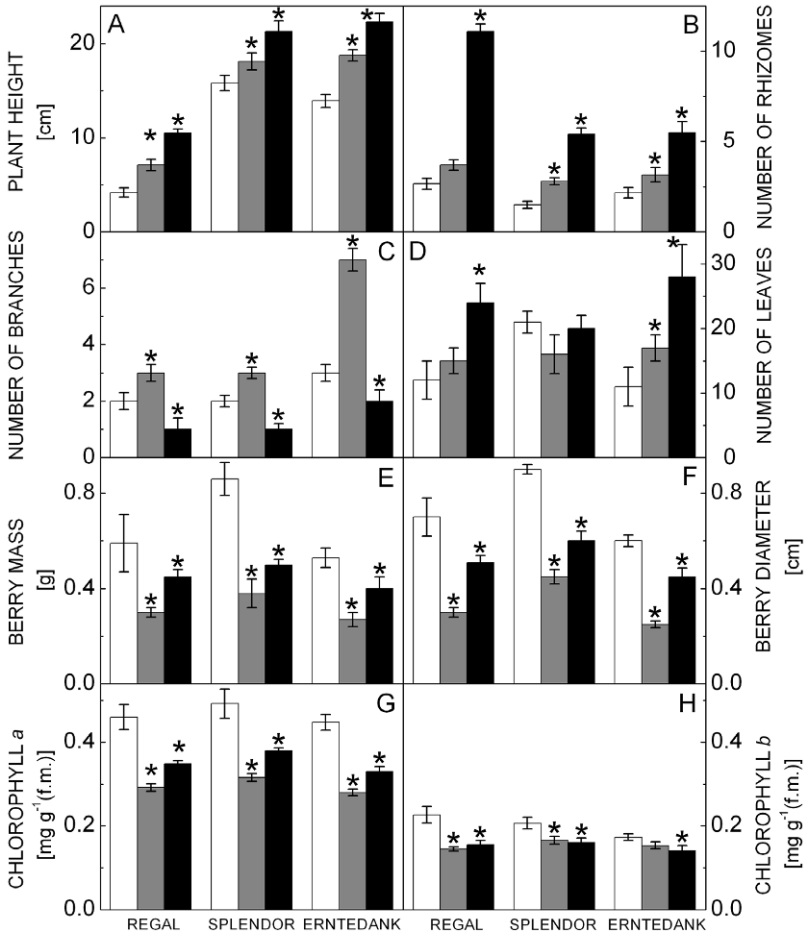

Fig. 2. Morphological characteristics (plant height - *A*, number of rhizomes per plant - *B*, number of branches per rhizome - *C*, number of leaves per branch - *D*, berry mass - *E*, berry diameter - *F*) and content of leaf chlorophyll *a* (*G*) and chlorophyll *b* (*H*) of lingonberry cultivars Regal, Splendor, and Erntedank obtained by three different propagation methods: stem cutting (control plants, *open bars*), node culture (*grey bars*), and leaf culture (*black bars*). Means ± SE, $n = 3$, * - values significantly different from the control at $P < 0.05$.

glutathione was also nearly the same in all SC plants. It increased significantly in NC and LC which corresponded also to decrease in GSSG (Fig. 3). Reduction potential (E_{hc}) of glutathione was calculated according to the formula of Schafer and Buettner (2001):

E_{hc} [mV] = -240 - (59.1/2) log ([GSH]^2/[GSSG]).

The SC plants possessed the least negative reduction potential of glutathione (from -235 mV in Erntedank to -242 and -245 mV in Regal and Splendor, respectively), while NC had the most negative values (-262 mV in Splendor, -270 mV in Regal, and -272 mV in Erntedank), and slightly less negative values were in LC

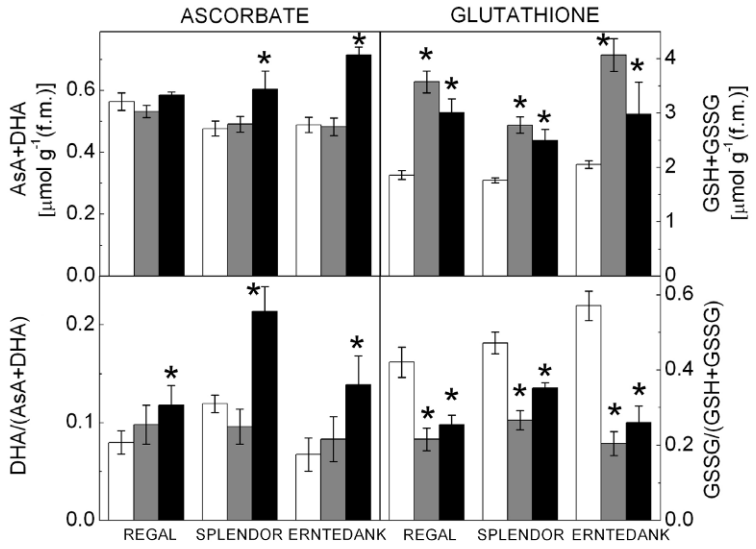

Fig. 3. Content of ascorbate + glutathione and of their oxidized species (DHA + GSSG) in leaves of three lingonberry cultivars (Regal, Splendor, and Erntedank) propagated by three different methods: stem cutting (control, *open bars*), node culture (*grey bars*), and leaf culture (*black bars*). Means ± SE, $n = 3$, * - values significantly different from the control at $P < 0.05$.

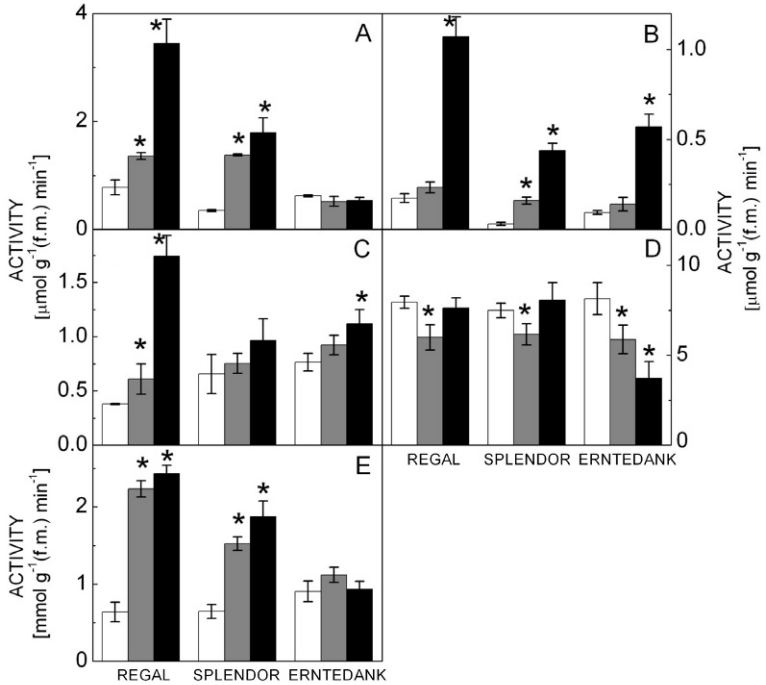

Fig. 4. Activities of enzymes of the ascorbate-glutathione cycle (ascorbate peroxidase - *A*, glutathione reductase - *B*, monodehydroascorbate reductase - *C*, dehydroascorbate reductase - *D*) and of catalase (*E*) in leaves of three lingonberry cultivars (Regal, Splendor, and Erntedank) propagated by stem cutting (control, *open bars*), node cultures (*grey bars*), and leaf cultures (*black bars*). Means ± SE, $n = 3$, * - values significantly different from the control at $P < 0.05$.

(from -254 to -265 mV).

Activities of the ascorbate-glutathione cycle enzymes and catalase in leaves differed among the cultivars and were affected by the propagation method (Fig. 4). In Regal and Splendor, APX activities in LC were 5 and 7 times higher than in SC, and in NC 1.5 to 5 times higher than in CS. The APX activity in Erntedank remained similar for all three propagation methods. The increase in APX corresponds in general to a higher content of the oxidized ascorbate species (DHA) (Fig. 3). GR activity in leaves was affected by propagation methods in a similar way as APX. The GR activity in LC was 5 - 10 times higher than in SC. The increase in GR corresponds to a decrease of the portion of GSSG in relation to the total glutathione content (Fig. 3). MDHAR activity showed a similar pattern as GR but the difference betweeen LC and SC was most striking in Regal and small in Splendor and Erntedank. DHAR activity exhibited a completely different pattern as compared to MDHAR, APX, and GR. In Regal and Splendor, it was slightly lower in NC as compared to SC and LC. In Erntedank, we observed very low DHAR activity. The low DHAR together with high APX in fact show a consistency with the DHA content in investigated plants (Fig. 3). CAT activity exhibited a similar pattern as APX activity with no difference in Erntedank.

The content of total soluble phenolics and other antioxidant compounds showed different (often opposite) patterns for fruits and leaves and was influenced by the propagation methods (Fig. 5). It was 5 - 10 times lower in fruits than in leaves (as calculated per fresh mass unit). The NC and LC decreased the phenolic content significantly as compared to SC to similar values in all cultivars. In fruits, the observed variations were smaller and the total phenolic content, in contrary with leaves, was enhanced by *in vitro* propagation in agreement with previous data of Foley and Debnath (2007).

The total anthocyanin content was not influenced by the propagation method except the observed significant decrease in leaves of Erntedank NC and LC plants. The

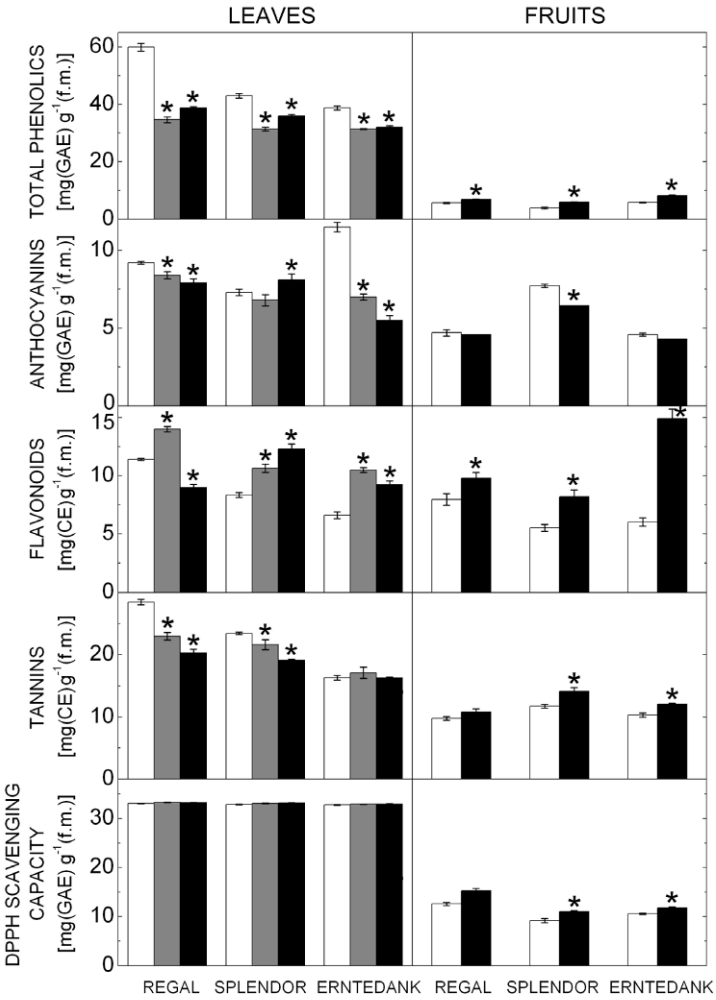

Fig. 5. The content of total phenolics, anthocyanins, flavonoids, and tannins, and radical scavenging capacity in leaves and fruits of three lingonberry cultivars propagated by different methods: stem cutting (control, *open bars*), node cultures (*grey bars*), and leaf cultures (*black bars*). GAE - gallic acid equivalent, CE - catechin equivalent. Means ± SE, $n = 3$, * - values significantly different from the control at $P < 0.05$.

Table 1. Effect of cultivar and propagation method (PM) on phenolic compounds [μg g^{-1} (f.m.)] in lingonberry cultivars. SC - stem cutting, LC - leaf culture, Cv - variance between cultivars for all propagation methods; PMv - variance between PM for all cultivars; Cv × PMv - variance for all propagation methods and all cultivars .

		Gallic acid		Catechin		Epicatechin		p-Coumaric acid		Quercetin	
		leaf	berry	leaf	berry	leaf	berry	leaf	berry	leaf	berry
Cultivar	Regal	0.201 c	0.177 a	2.328 c	0.968 a	0.632 b	0.111 b	0.089 b	0.051 b	3.689 a	0.124 a
	Splendor	0.331 b	0.163 a	2.153 b	0.857 b	0.576 c	0.134 a	0.124 a	0.047 b	2.613 c	0.107 b
	Erntedank	0.225 a	0.168 a	3.316 a	0.961 a	0.729 a	0.082 c	0.083 b	0.102 a	3.508 b	0.120 a
PM	SC	0.282 a	0.154 b	3.149 a	0.782 b	0.706 a	0.072 b	0.099 a	0.063 b	3.505 a	0.095 b
	LC	0.223 b	0.185 a	2.716 b	1.075 a	0.585 b	0.146 a	0.098 a	0.070 a	3.035 b	0.139 a
P values	Cv	<0.0001	0.3106	<0.0001	0.0011	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0004
	PMv	<0.0002	0.0005	<0.0001	<0.0001	<0.0001	<0.0001	0.8347	0.0031	<0.0001	<0.0001
	Cv × PMv	0.0077	0.9271	0.0108	<0.0001	<0.0001	<0.0001	0.4257	0.0104	0.0716	0.1140

anthocyanin content was nearly twice lower in fruits than in leaves. The total flavonoid content was approximately the same in leaves and fruits and it was significantly higher in fruits of the *in vitro*-derived plants especially for Erntedank. Total tannin content was similar and was approximately twice higher in leaves than in fruits. The total DPPH radical scavenging capacity was also twice higher in leaves than in fruits. In leaves, it was not affected by the method of propagation whereas in fruits of the plants obtained from tissue culture, the radical scavenging capacity was ~10 % higher as compared to SC.

Phenolic compounds identified in leaves and berry extracts by HPLC are shown in Table 1. Catechin, epicatechin, gallic acid, *p*-coumaric acid, and quercetin were identified in the extracts according to their MS/MS spectra following the breakdown of sugar-phenolic esters. Molecular masses of sugar moieties attached to quercetin were tentatively identified and confirmed with the literature (Anttonen *et al.* 2006, Ek *et al.* 2006). At least four quercetin derivatives were tentatively identified in the extracts. Quercetin-3-O-galactoside, quercetin-3-O-glucoside, and quercetin-3-O-arabinoside were unambiguously detected in fruit extracts whereas in leaves, quercetin-3-O-rutinoside was also identified in addition to the other three derivatives. Quantification of total quercetin and other identified compounds was done using standard calibration curves and peak areas. In fruits, the content of identified phenolics was higher in *in vitro*-derived plants except for *p*-coumaric acid content which was almost the same in fruits of *in vitro*- and *ex vitro*-derived plants. The trend was opposite in leaves and significantly higher content of identified compounds was observed in SC leaves than NC and LC, except for *p*-coumaric acid.

Discussion

The controlled morphogenesis with initiation of new meristems in plants can be forced by tissue culture techniques. By adding a cocktail of plant hormones, differentiated cells can be reset to start formation of new shoots or roots. Hormones act as triggers *via* signalling events followed by a long chain of metabolic steps occurring after binding them to receptors and leading generally to modulation of the redox state. The morphogenetic phenomena are directly controlled by metabolism and, in particular, by the pairs of reduced and oxidized compounds, the most important of which are ascorbate and glutathione. We explore a possibility that the differences in the morphogenetic process in tissue culture plants may be directly related to the redox state of glutathione and ascorbate. This modulation is linked to the concentration of reactive species such as hydroxyl radicals, superoxide anion, hydrogen peroxide, and monodehydroascorbate (ascorbate free radical, AFR).

The direct involvement of radical species in morphogenetic process was shown (Jana and Shekhawat 2012), *e.g.*, in relation to loosening of cell walls (Cárdenas 2009, Mülle *et al.* 2009). Reactive oxygen species participate in cell expansion during the morphogenesis in particular by affecting the activity of calcium channels required for polar growth (Carol and Dolan 2006). As an example, prooxidant and antioxidant enzymes can control development by establishing necessary concentrations of superoxide and H_2O_2 for regulation plant cell expansion through the activation of Ca^{2+} channels (Foreman *et al.* 2003). Concrete mechanisms of directing morphogenesis may involve the control of polarized cell growth in plants by Rho-like small GTPases (ROPs) through plant-specific pathways involving the regulated release and scavenging of reactive oxygen species (Uhrig and Hülskamp 2006). The enzymes modulating content of reactive oxygen species, such as the alternative oxidase of mitochondria, are directly involved in determination of the rate of growth, branching, and other morphogenetic

events (McNulty *et al.* 1988, Hilal *et al.* 1997, Fiorani *et al.* 2005, Frederico *et al.* 2009). However, the enzymes of the ascorbate glutathione cycle plus CAT, that directly determine the redox states of ascorbate and glutathione and the concentrations of AFR and H_2O_2, are the most important in regulating morphogenesis (Gupta and Datta 2003, Vatankhah *et al.* 2010).

This study explores antioxidant metabolism in relation to the lingonberry cultivars strongly differing in their basic features that include height, branching, and berry size, and the methods of their propagation. Despite certain differences among the cultivars, the leaves of plants obtained from the *in vitro* culture showed significantly higher antioxidant enzyme activities (except for DHAR). This is in line with the observation that the total radical scavenging capacity was enhanced in both berries and leaves of the *in vitro* propagated plants. Another notable observation of this study is the strong difference in morphological properties of the cultivar Enterdank from two other studied cultivars. First, the difference in the height of Erntedank plants obtained from stem cutting and tissue culture is the highest among all cultivars. The percent change in the number of branches per rhizome is significantly higher in Erntedank. The mass and diameter of berries in Erntedank is not much changed compared to other cultivars whereas the percent change from different cultivation methods is almost similar. Total soluble phenolics, tannins, and flavonoids were found to be enhanced in fruits of plants obtained by the *in vitro* propagation method whereas in leaves, these metabolites were higher in SC plants except for flavonoid content which was higher in leaves of NC and LC plants. The total anthocyanin content was not significantly different in fruits of differentially propagated plants.

Generally, we observe lower total phenolic, anthocyanin, and tannin content in leaves and higher in fruits. Reverse changes in antioxidant metabolites in fruits as compared to vegetative parts in relation to the method of propagation also correspond to lower number of berries, their lower mass, and size (Fig. 2). In general, this means that tissue culture propagation enhances growth and metabolism in vegetative parts of plants, whereas the increased antioxidant properties of smaller berries can be explained by a higher proportion of berry coat which is enriched by antioxidant compounds.

The cellular redox state is considered as an important determinant for plant growth and development. Schafer and Buettner (2001) suggested that the resulting action of several redox couples which are able to interconvert between oxidized and reduced forms can trigger major metabolic events and determine morphogenetic processes in plants. The displacement of equilibrium between the reduced and oxidized forms of such compounds as ascorbate and glutathione can essentially affect tissue patterning and morphogenesis (Mitrovic *et al.* 2012, Talukdar 2012). The synergistically interacting redox couples of glutathione (GSH/GSSG) and ascorbate (AsA/DHA + AFR) are defined as the best indicators of the overall cellular environment (Schafer and Buettner 2001). The involvement of GSH in cell division processes is proven by its stimulation of *Arabidopsis* root growth (Sanchez-Fernandez *et al.* 1997). Whereas GSH favours cell division and proliferation through a direct involvement in the cell cycle machinery, GSSG content is related to differentiation (Schafer and Buettner 2001, Stasolla *et al.* 2004). We can see from the obtained data that more reduced glutathione pool in the *in vitro*-propagated plants results in their increased height and increased number of rhizomes and leaves per branch but decreased number of branches per rhizome. Possible correlations between the growth and morphological characteristics of the studied cultivars may involve direct regulation of cell division and differentiation by the redox state of the ascorbate and glutathione. Ascorbate and glutathione reduction pairs may have different roles in regulation of metabolism as they have different redox potentials. It was shown, for example, that they both are needed to keep iron in the reduced form in plant hemeproteins (Igamberdiev *et al.* 2006, 2011). While glutathione has direct influence on morphogenetic phenomena, ascorbate also has effects on elongation *via* influence on cell walls. It is shown that high AsA/DHA ratio observed in stem cutting plants ensures the reactivation of meristematic cells (Stasolla and Yeung 2006). Glutathione has high symplastic and low apoplastic pool whereas ascorbate pools are high both in symplast and in apoplast. Apoplastic ascorbate (being reduced from the cytosolic side) participates in reactions of phenolic compounds, *e.g.*, in scavenging phenoxy radicals that participate in lignin formation (Horemans *et al.* 2000). Higher total content of ascorbate and its lower reduction correlate with increased height, higher number of branches and rhizomes per plant, and higher leaf number per branch (Fig. 2). Also, more reduced ascorbate pool in the SC plants corresponds to higher phenol, anthocyanin, and tannin content which may be related to the reactions in apoplast in which ascorbate participates (Horemans *et al.* 2000).

The actual glutathione redox potential is related to $[GSH]^2/GSSG$. Thus, unlike many other redox couples (*e.g.*, $NADP^+/NADPH$), the glutathione redox potential depends on and can be influenced by absolute content as well as by changes in GSSG relative to GSH (Mullinieaux and Rausch 2005, Meyer 2008). Even if the GSH/GSSG ratio remains unchanged, decreases in glutathione content alone will lead to an increase in redox potential. More negative redox potentials in leaves of the NC and LC plants correspond to their increased height, highly increased number of branches and rhizomes per plant, and smaller mass and diameter of berries. Less evident is correlation with number of leaves per branch (not statistically different in Splendor) and with the number of secondary branches which in all cultivars is higher in the NC and lower in the LC as compared to the SC plants. It may be related to more negative redox potentials in the NC plants as compared to the LC plants. Less negative potentials lead to differentiation (Schafer

and Buettner 2001) which corresponds to deposition of more biomass in berries in the SC plants, their lower height and a higher number of secondary branches as compared to the LC plants (Fig. 2).

The importance of glutathione pool size and its redox potential for determination of cell division, growth, and even apoptosis was mentioned in many works (reviewed in Noctor *et al.* 2012). The quiescent parts of plants such as root quiescent center, and cells in organs such as seeds maintain a highly oxidized intracellular state, in particular reflected in low content of reduction of the glutathione pool (Kranner *et al.* 2006). Auxin accumulation in the root stem cells is dependent on the oxidized status of the cells (Jiang and Feldman 2010). The reduction of glutathione content results in a non-functional root meristem whereas the shoot meristem is largely unaffected (Vernoux *et al.* 2000). A subsequent increase in the total cellular GSH pool is essential for the cells to progress to cell division (Diaz-Vivancos *et al.* 2010). Glutathione synthesis is also required for pollen germination and pollen tube growth (Zechmann *et al.* 2011). Certainly, the reduction potentials of glutathione determined both by its reduction level and concentration (Schafer and Buettner 2001) can give only an indirect indication of its influence because its direct effect should be observed in relation to its potential in meristematic cells.

The data presented in this study show an important connection between the pool size and reduction levels of ascorbate and glutathione, activities of enzymes of ascorbate-glutathione cycle, and the parameters of growth and differentiation of lingonberry plants.

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