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ARTICLE

Studies on elemental composition and antioxidant capacity in callus cultures and native plants of *Vaccinium myrtillus* L. local populations

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ABSTRACT The biological and medical effects of bilberry fruit (*Vaccinium myrtillus* L.) are mainly due to high anthocyanin content of tissues. Calli containing anthocyanins, derived from bilberry plants, may represent a potential source of natural colouring matter, pharmaceutical and natural antioxidants. In the present study we investigated the occurrence of differences in elemental composition and antioxidant capacity of the three local populations of mountain bilberry collected in the western region of Romania (Arieseni, Retezat and Sebes Valley) in order to compare the anthocyanin production of plant and callus tissues originated from various plant populations. It was found that K, Fe and Zn content was higher in calli than in intact plant leaves. The excess of latter two microelements, Fe and Zn can induce oxidative stress, and, as a result of the accumulation of reactive oxygen species, various antioxidant mechanisms. The total antioxidant capacity of callus cultures determined by FRAP method (ferric reducing antioxidant power) could be enhanced as a function of increasing adenine sulphate (AS) concentration in the culture medium and it depended on the origin of mother plants. The leaves of intact plants contained higher amount of total non-protein sulfhydryl groups than calli, and the decrease was especially significant in tissue cultures originated from the Retezat region. In contrast, depending on the AS concentration, the anthocyanin content could increase in callus cultures. The tissues originated from various populations exhibited different AS concentration optimum. This suggests that bilberry callus cultures can be a suitable source of the anthocyanins.
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KEY WORDS

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Lowbush bilberry belongs to the genus *Vaccinium* (Ericaceae family), which contains about 400 species. *Vaccinium myrtillus* L. is native to Europe and North America. This plant is a component of ground layer vegetation in forests of cold and temperate climate zones and forests at higher elevations in Southern Europe, and it has an important role in the nutrient fluxes of natural ecosystems. It was also found that the elemental composition and the accumulation of phenolic compounds in bilberry leaves were suitable indicators of heavy metal stress and it could effectively indicate the elemental content of soil (Mróz and Demczuk 2010).

Bilberry is considered to be an important nutritional resource for humans. The fruits and leaves are rich in phenolic compounds, especially in anthocyanins and other antioxidants (Martz et al. 2010). It was found that the antioxidant capacity of blueberry (*Vaccinium corymbosum* L.) cultivars could be influenced by the genotype, but other factors (e.g. growing season, location, the age of plants, storage condition of samples) can also affect these parameters (Piljac-Žegarac et al. 2009). Over the years, a series of chemical analyses have revealed these health-beneficial compounds in bilberry fruits, however, the underlying genetic diversity and the variation in biochemical composition between populations and *in vitro* callus cultures, remain to be thoroughly investigated. The aim of the present study was to investigate the differences in the elemental composition, total antioxidant capacity, anthocyanin and total non-protein thiol content of intact leaves and calluses derived from various populations of bilberry collected in the western part of Romanian mountains.

In vitro propagated callus cultures can become an alternative to plants grown in their native environment due to the fact that under controlled conditions, plant tissues can produce significant amounts of metabolites of interest. Moreover, the antioxidant activity and the content of macro- and microelements may represent parameters that indicate the occurrence of somatic variability in the callus, this fact being important in selecting the cell lines of interest.

The hormone balance and chemicals applied in the culture medium are important for the selection of callus lines with high antioxidant capacity. Therefore, we also would like to

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determine whether the application of adenine sulphate (AS) in the culture medium favors growth and antioxidant capacity of callus cultures established from various native populations of bilberry.

Materials and Methods

Plant material representing native populations of bilberry was collected from three sites of Carpathian mountains in western Romania, in Arieseni, Retezat and Sebes Valley districts. Because of the difficulties of the representative sampling for elemental analysis the fully expanded leaves of naturaly-ly growing plants were collected two times in June 2011. Five plants were selected each time and were transported to laboratory in an icebox. Then the leaves were detached and after washing with double distilled water, they were stored at -80°C until analysis (Kovacheva et al. 2000). To obtain calluses, different types of explants were taken from various tissues of bilberry plants, originated from the three different mountain locations. After surface sterilization the explants were inoculated on the Woody Plant Medium (WPM) (Lloyd & McCown's Woody Plant Medium, PhytoTechnology Laboratories, Lenexa, Kansas, USA), supplemented with 5,24 µM 1-naphthylacetic acid (NAA) and 5 µM benzylaminopurine (BAP) and with different concentrations of AS (Sigma, Chemical Co., St. Louis, Missouri). Concentrations of AS were 99 µM (40 mg/l), 148,51 µM (60 mg/l) and 198,01 µM (80 mg/l). The calluses used in this study were analyzed after three subcultures on the same type of culture medium and under the same hormonal influence.

Determination of macro- and microelement content

The content of macro- (K, Ca, Mg) and microelements (Cu, Zn, Fe, Ni) as well as some heavy metals (Cr, Cd, Pb) in *Vaccinium* calluses and mother plant leaves was determined by atomic absorption spectrometry (AAS) with a Hitachi Z-8200 spectrophotometer (Tokyo, Japan). For each sample, 100 milligrams of dried plant or dried callus tissues were used. Plant material was homogenized and placed in test tubes containing 5 ml of concentrated HNO_3 and 4 ml of 30% H_2O_2 at 200°C for 2 hours. For the determination of metal content, 3 replicas were taken in each experiment for each sample. Metal content in the samples are given in µmol g^{-1} dry mass (DM).

Preparation of samples for biochemical assays

Fresh plant material (0.3 g) was homogenized with 1.2 ml of cool 0.1 M phosphate buffer (K_2HPO_4, pH 7.6) containing 0.1 mM EDTA, and centrifuged for 10 min at 12,000g. Then the supernatant was used for the detection of total antioxidant capacity and total non-protein thiol assays. Non-protein sulfhydryl groups were expressed in glutathione (GSH) equivalents.

Determination of total antioxidant capacity (FRAP)

The total antioxidant activity was determined by FRAP method (Ferric Reducing Activity of Plasma or Ferric Reducing Antioxidant Power) which measure the ferric ion reducing capacity of the cytoplasm (Benzie and Strain, 1996). The plant extract was prepared and the measurements were done according to the modification of Varga et al. (2000) and Szőllősi and Varga (2002). The reaction mixture contained 50 µl plant extract and 1.5 ml FRAP reagent (300 mM acetate buffer, pH 3.6, 10 mM tripyridyltriazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3$ in ratio 10: 1: 1). Ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion reduction at low pH causes a coloured ferrous-tripyridyltriazine (Fe^{2+}-TPTZ) complex to form. The absorbance was determined with spectrophotometer at 593 nm. The total antioxidant capacity was expressed in units of µmol g^{-1} fresh weight (FW).

Determination of total non-protein thiol content

Total non-protein thiol content was measured using the method of Sedlak and Lindsay (1968). 125 µl of plant extract and 0.5 ml of 5% (w/v) trichloroacetic acid (TCA) were mixed and centrifuged for 10 min at 10,000g. Then the supernatant was used for the measurement by adding 0.4 M Tris buffer (pH 8.9) and 5,5'-dithio*bis* (2- nitrobenzoic acid) (DTNB) and the absorbance was detected by the spectrophotometer at 412 nm. Data are expressed in µmol GSH g^{-1} fresh weight (FW).

Analysis of anthocyanin compounds

Anthocyanin compounds were extracted from 250 mg of fresh callus at 4°C, using 2 ml concentrated methanol (MetOH, 99% v/v) acidified with 1N hydrochloric acid (HCl) in 1:1 ratio according to the assay of Lange et al. (1971). After extraction, the samples were centrifuged for 20 minutes at 12,000g and the supernatant was analyzed with spectrophotometer at 479 nm.

To estimate the anthocyanin-concentration of plant tissues by measuring the absorbance, the anthocyanin contents are expressed in cyanidin-3-glucoside equivalents (mg) and calculated for 1 g fresh weight. The calculation of the concentration was based on Lambert-Beer's law using a molar extinction coefficient of 2.95×10^4.

Statistics

The statistical analysis of results was carried out using STATISTICA 9.0 software. First we executed two-way ANOVA to detect the effect of population and/or treatment on several parameters. Then non-parametric test (Kruskal-Wallis ANOVA) was used to test the differences of means. In order to deter-

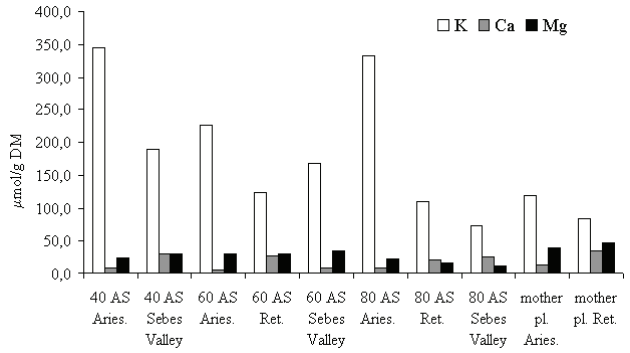

Figure 1. The macroelement (K, Ca, Mg) content in the calluses and the leaves of mother plants of *Vaccinium myrtillus* L. originated from Arieseni, Retezat and Sebes Valley districts. The culture medium was supplemented with 40, 60 and 80 mg l^{-1} adenine sulphate (AS). Data are given in µmol g^{-1} DM (dry mass).

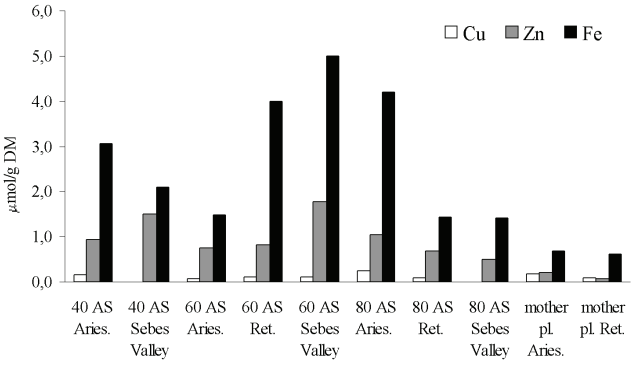

Figure 2. The microelement (Cu, Zn, Fe) content in the calluses and the leaves of mother plants of *Vaccinium myrtillus* L. originated from Arieseni, Retezat and Sebes Valley districts. The culture medium was supplemented with 40, 60 and 80 mg l^{-1} adenine sulphate (AS). Data are given in µmol g^{-1} DM (dry mass).

mine the relationship between the biochemical parameters, a non-parametric analysis of correlation (Spearman's Rank Order Correlation) was used. Data are given in mean values ± standard deviation (SD) and calculated for fresh weight (FW). Level of significance was generally $p<0.05$.

Results

The content of macro- and microelements

The amount of macroelements of the *Vaccinium* calluses derived from several populations had a high diversity and ranged from 73.6 to 346 µmol g^{-1} DM for potassium, 6.8-35 µmol g^{-1} DM for calcium and 11.6-47 µmol g^{-1} DM for magnesium (Fig. 1). The potassium level was usually higher in the calluses than in the mother plants that were regarded as

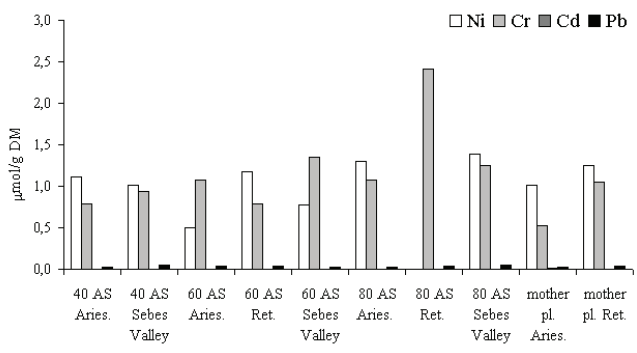

Figure 3. The heavy metal (Ni, Cr, Cd, Pb) content in the calluses and the leaves of mother plants of *Vaccinium myrtillus* L. originated from Arieseni, Retezat and Sebes Valley districts. The culture medium was supplemented with 40, 60 and 80 mg l^{-1} adenine sulphate (AS). Data are given in µmol g^{-1} DM (dry mass).

control (119.5 and 84.1 µmol g^{-1} DM in the samples derived from Arieseni and Retezat, respectively), while the calcium and magnesium content of the mother plants was generally higher than those of the calluses.

Increasing concentrations of AS in the culture medium did not enhance the macroelement content of callus tissues, in contrast, the highest AS concentration (80mg l^{-1}) may result in a decline in K content in certain cell lines.

Copper level of the calluses were similar to those of the mother plants and were very low (0-0.25 µmol g^{-1} DM), but zinc concentrations of the calluses were 5-9-times higher than those of the control (0.06-1.8 µmol g^{-1} DM). At the same time, calluses showed very high iron levels compared to control (0.6-5 µmol g^{-1} DM, Fig. 2).

The amounts of Ni (essential element) and Cr were very low and the concentrations of two other non-essential heavy metals, Cd and Pb were close to zero in all samples (Fig. 3).

The total antioxidant capacity (FRAP)

Since analysis of variance (ANOVA) revealed that the population and the treatment had an effect on the FRAP values, the effect of population being stronger, ($F_{2, 26}$= 20.62, $p<0.001$; Table 1), we compared the data of the different populations (V1= Arieseni, V2= Retezat, V3= Sebes Valley) grown on increasing concentration of AS.

We found significant differences between the populations only at 60 AS treatment (Fig. 4). Surprisingly, FRAP did not show significant correlation neither with free, non-protein thiol level nor the macro- or microelement content.

Total non-protein thiol content of tissues

We found that both the population type and the treatment have an effect on the accumulation on non-protein thiols

Table 1. Results of two-way ANOVA testing the effect of population type and treatment on ferric reducing capacity (FRAP) and total non-protein thiol (GSH) content in *Vaccinium myrtillus* L.

Effect	FRAP	GSH
Population	$F_{2, 26}$ = 20.62***	$F_{2, 26}$ = 21.25***
Treatment	$F_{3, 26}$ = 3.84*	$F_{3, 26}$ = 31.62***
Popul. x treatm.	$F_{6, 26}$ = 2.83*	$F_{6, 26}$ = 2.63*

Significance levels are indicated by * and *** representing p< 0.05 and p< 0.001.

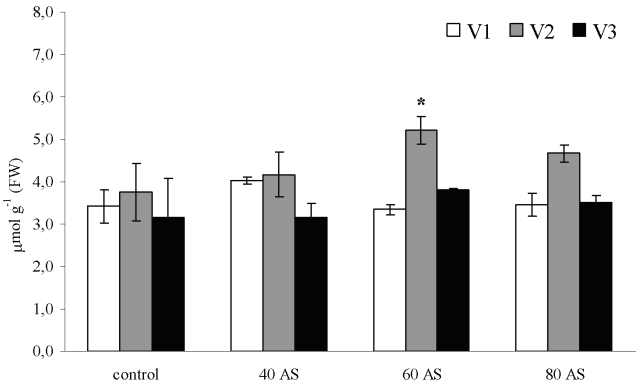

Figure 4. The total antioxidant capacity (FRAP) of the calluses and mother plants (control) of *Vaccinium myrtillus* L. originated from Arieseni (white column, V1), Retezat (grey column, V2) and Sebes Valley (black column, V3) districts. The culture medium was supplemented with 40, 60 and 80 mg l^{-1} adenine sulphate (AS). Data are given in µmol g^{-1} FW (fresh weight). Mean ±SD (n=3). Asterisk (*) refer to significant difference between control and treated plants within the same population, at p<0.05.

(Table 1). After further analysis, significant differences were found among the populations in the control samples. Moreover, callus cultures exhibited a decrease in total non-protein thiol content in all populations (Fig. 5) and non-protein thiol concentrations in tissues growing on 40-80 mg l^{-1} AS were rather similar to each other. At the same time, within V2 population, all types of calluses had much lower free SH levels than the intact plant samples. We found significant correlations between total non-protein thiol content of tissues, and elemental composition, a positive correlation coefficient for Mg (r= 0.59, p< 0.001) and negative values for Zn (r= -0.70, p< 0.001) and Fe (r= -0.60, p< 0.001).

Total anthocyanin content

In all calluses and the control plants remarkable differences were found between V2 and V3 populations (Fig. 6). We also found relatively strong positive correlation between total anthocyanin content and FRAP values (r= 0.60, p< 0.001; Fig. 7). It was also found that depending on the genotype, the anthocyanin contents could increase transiently (V1- white column and V3- black column) as a function AS concentration.

Discussion

In our experiments it was revealed that elemental composition of callus cultures can show significant differences from the tissues of mother plants and in some cases there were significant differences between the tissues originated from different populations.

Increasing the amount of AS (60-80 mg l^{-1}) in the culture medium causes the accumulation of iron in bilberry callus. The tissues also accumulate zinc in smaller amounts, and the

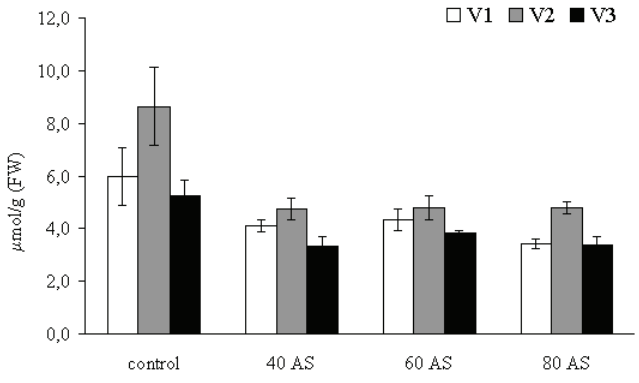

Figure 5. The total non-protein thiol content of the calluses (expressed in glutathione equivalents) and mother plants (control) of *Vaccinium myrtillus* L. originated from Arieseni (white column, V1), Retezat (grey column, V2) and Sebes Valley (black column, V3) districts. The culture medium was supplemented with 40, 60 and 80 mg l^{-1} adenine sulphate (AS). Data are given in µmol g^{-1} FW (fresh weight). Mean±SD (n=3).

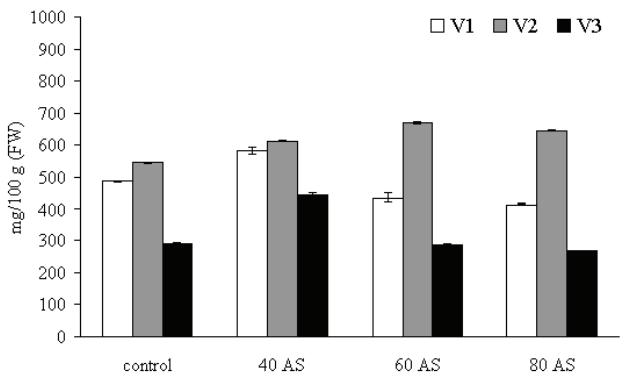

Figure 6. The total anthocyanin content of the calluses and mother plants (control) of *Vaccinium myrtillus* L. originated from Arieseni (white column, V1), Retezat (grey column, V2) and Sebes Valley (black column, V3) districts. The culture medium was supplemented with 40, 60 and 80 mg l^{-1} adenine sulphate (AS). Data are given in µmol g^{-1} FW (fresh weight). Mean±SD (n=3).

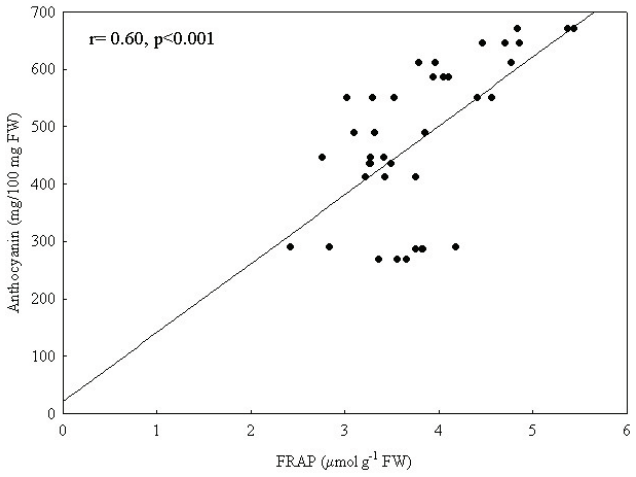

Figure 7. Spearman's Rank Correlation between the total anthocyanin content and FRAP in *Vaccinium myrtillus* L. tissues.

zinc content in callus cultures is higher than in the mother plant. This phenomenon makes the callus tissues usable in treatments to combat the deficit in iron and zinc of food, in form of food or feed dietary supplements.

Regarding the heavy metals, chromium content reaches slightly higher values in the callus tissues than in the mother plants. Growing callus on culture medium which is supplemented with 40 mg l^{-1} and 60 mg l^{-1} AS does not change the content of these non-essential heavy metals compared to the mother plants.

The addition of AS to the culture medium may increase the total antioxidant capacity of bilberry calluses, the effect was most significant in tissues of V2 and V3 populations treated with 60 mg l^{-1} AS.

The pool of non-enzymatic antioxidants and antioxidant enzymes is induced very frequently by oxidative stress itself (Csiszár et al. 2004). However, the non-protein thiol content of calli are lower than those of intact plant tissues suggesting that the cells in tissue culture are probably exposed to a slight oxidative stress due to the relatively high concentrations of plant hormones in the culture media or due to the excess of Fe and Zn in callus tissues. Since glutathione constitutes the highest portion of free, non-protein thiols in plant tissues, we can suppose that GSH content may be exhausted in these tissues as it was found in wheat roots exposed to heavy metal stress generating reactive oxygen species (Tari et al. 2002). The synthesis of phenolic compounds such as anthocyanins is also induced by oxidative stress caused by heavy metals (Mróz and Demczuk, 2010) or H_2O_2-generating chemicals such as salicylic acid (Szepesi et al. 2008).

The anthocyanin content of bilberry calluses is greater than that of the mother plants leaves, under *in vitro* culture conditions used. We suggest that both heavy metal stress and application of AS in the culture medium favors the biosynthesis of these important components with therapeutic value and the accumulation of anthocyanins is a consequence of the generation of reactive oxygen species in callus tissues growing on culture medium supplemented with AS.

Abbreviations

NAA: 1-naphthylacetic acid; BAP: benzylaminopurine; AS: adenine sulphate.

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