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Variation of Polyphenol Content and Antioxidant Activity in Some Bilberry (*Vaccinium myrtillus* L.) Populations from Romania

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Abstract: Fruits of bilberry (*Vaccinium myrtillus* L.) are valued mainly for their nutraceutical properties, and are among the fruits with the highest antioxidant activity due to their high content of phenolic compounds. The aim of this research was to assess the total polyphenol content and antioxidant activity of fruits in six wild bilberry populations from two regions of Romania over three years. The total polyphenol content was determined according to the Folin–Ciocalteu modified method, while the antioxidant activity was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay. The Padis and Raul Lung populations registered the highest values of polyphenol content and antioxidant activity, as such the fruits of these bilberry populations could be considered potential sources of antioxidants for direct consumption or for use as ingredients for food products or food supplements. Significant variation of total polyphenol content and antioxidant activity was observed both between populations from the same region and from different regions. The low level of broad sense heritability for total polyphenol content and antioxidant activity associated with the high effects of year, and population–year interaction indicates that the accumulation of polyphenols in bilberry fruits is influenced by changes in environmental conditions.

Keywords: bilberry; polyphenol content; antioxidant activity; DPPH radical scavenging

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1. Introduction

Bilberry (*Vaccinium myrtillus* L.), also called as European blueberry, is a deciduous low growing shrub native to northern Europe, also found in other different parts of Europe [1] including Romania, being widespread in the semi-mountainous regions.

Bilberry produces dark purple edible fruits and grows on acidic soils in meadows and moist coniferous forests, under moderate shade and humid ground conditions [2]. Bilberries are considered the most valuable berries, being called the fruits of the mountains that capture much of the solar energy from the high plateaus that they turn into healthy food. The fruits of bilberry can be consumed fresh, frozen, or dried, as well as in different processed forms, such as juices, jams, wine analogues, liqueurs, or food supplements [3]. Considering that during the processing of fruits, the levels of phenolics are affected, the consumption of fresh fruits is more beneficial, but processed products are valuable sources of phenolic compounds all year long [4].

Bilberry is classified as a Class 1 plant by the American Herbal Products Association, which means that it can be safely consumed when used properly. The recommended daily

doses are 20–60 g of dried fruit and 160–480 mg of extract powder [5]. The nutritional value of bilberries is about 399 kcal/100 g, and the main chemical compounds are: carbohydrates (94.5%); proteins (3%); ash (1.5%); and fat (1%) [6]. Bilberry fruits are valuable sources of various micronutrients and phytochemical compounds with important health benefits, such as: phenolic compounds [7,8]; vitamins [9]; sugars [10,11]. Additionally, bilberry fruits contain important amounts of carotenoids, higher than cultivated blueberry [12].

The increase of consumer demand for bilberry fruits is related to their high content of different phenolic compounds with various health-protective actions [3]. The changes of nutritional and chemical composition of bilberry fruits is a consequence of both genotype and environment effects. Several studies demonstrated that fruits growing in northern latitudes have higher phenolic contents compared with those from southern latitudes [13,14]. Also, in conditions with high photosynthetic active radiation, the total phenolic content and antioxidative capacity of bilberry fruits increased significantly [15]. In sunny sites without damaged topsoil, the fruits contain higher amounts of phenolic compounds [16], as in the case of higher altitudes [17].

The main phenolic compounds of bilberries are flavonoids, tannins, phenolic acids, but the most important are the anthocyanins [18]. Bilberry is considered the richest natural sources of anthocyanins. These compounds give the blue/black color of the fruits, and are considered the most biologically active compounds in the bilberry, being responsible for many health benefits of bilberry [19]. The concentration of anthocyanins increases approximately 70 times from green to ripe fruits [20], due to changes that cause a general decrease in the synthesis of other polyphenols during blueberry maturation and an intensification of anthocyanin synthesis [21]. The quantity of the anthocyanins in bilberry fruits is more than twice that of the fruits of cultivated blueberry [22,23]. Increased altitudes associated with progressive decreases of temperature and increased light intensity, have been reported to have a positive effect on anthocyanin accumulation [24].

The polyphenol-rich extract of bilberry fruits has an inhibitory activity towards the α-glucosidase enzyme, thus is useful in controlling type 2 diabetes [25]. Extracts of bilberry have also shown to have a protective effect on visual function during retinal inflammation [26] and antiproliferative activity in human cancer cell lines after in vivo treatment [27]. Bilberry extracts also showed inhibitory effects against lipid peroxidation and preventive activity to oxidative DNA damage [28].

Compared to the cultivated genotypes of *Vaccinium corymbosum*, bilberry had approximately two to three times higher antioxidant capacity [29,30], and higher concentration of bioactive components [31–33], with great positive impacts on health [34].

Antioxidants are necessary to prevent and protect against the action of unstable free radicals, which cause damage to different body biomolecules and lead to cancers, heart diseases, and many associated health problems [28]. A diet rich in antioxidants can be an effective way for reducing the incidence of these illnesses, because antioxidants may block the attack of free radicals. Natural antioxidants are preferred by the consumers, because they are safe compared to the synthetic antioxidants [35]. Considering that most natural antioxidants have reactive hydrogen atoms which act as reductants, the DPPH procedure is a good estimation of the standard antioxidant profile [36].

Given that bilberry is widespread in the Banat Mountains, where important quantities of fruits are harvested, and that the fruits are highly sought after and appreciated by the consumers, we believe that it is warranted to study the quality of these fruits. The aim of this research was to assess the total polyphenol content and antioxidant activity of fruits in different bilberry populations, in order to obtain information regarding their nutraceutical potential. The study was conducted on six wild bilberry populations from two regions of Romania during 2018–2020. To our knowledge, this study was the first to investigate the antioxidant potential of bilberry population from Banat Mountains.

2. Materials and Methods

2.1. Plant Material

Fully ripe and undamaged bilberry fruits were harvested at the beginning of August during 2018–2020 from five different sites (Muntele Mic, Raul Lung, Semenic, Sadova Noua, Cuntu) of the Banat Mountains and one site (Padis) from the Apuseni Mountains. The berries were handpicked, bagged, and kept refrigerated (approximately 10 °C) during transport. After that, the fruit samples were stored at −20 °C prior to processing.

Soil samples were collected from the O-layer in all sites. According to the data from Table 1, the soil in all sites was extremely acidic, with pH values ranging from 3.95 to 4.95. The total nitrogen content had low amplitude compared to phosphorus and potassium contents.

Table 1. Environmental characteristics of sites for bilberry populations.

Population	Altitude (m)	Latitude	Longitude	Soil Parameters			
				pH	N (%)	P (ppm)	K (ppm)
Muntele Mic	1522	45°21′36″ N	22°27′54″ E	4.18	0.65	20.56	181
Raul Lung	1297	45°16′19″ N	22°29′05″ E	3.95	0.70	51.32	308
Semenic	1417	45°10′54″ N	22°03′10″ E	4.03	0.89	17.66	319
Cuntu	1439	45°18′02″ N	22°29′54″ E	4.24	0.84	56.54	366
Sadova Noua	750	45°15′28″ N	22°22′57″ E	4.28	0.90	42.64	454
Padis	1313	46°36′05″ N	22°44′03″ E	4.95	0.82	62.48	295

2.2. Preparation of Extracts

The bilberry fruits were air dried at 30 °C, subsequent the dry material was ground to a fine powder using a grinder (GM 2000; Grindomix; Retsch Technology GMbH, Haan, Germany). The powdered material (1 g) was extracted with 10 mL 60% ethanol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 30 min using an ultrasonic water bath (FALC Instruments, Treviglio, Italy) [37–39]. Extracts were then filtered using Whatman membrane filters 0.45 µm, 30 mm (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and stored for 24 h at 2–4 °C, until subsequent chemical analyses.

2.3. Assessment of Total Polyphenol Content

Total polyphenol content was determined according to the Folin–Ciocalteu modified method [40]. An amount of 0.5 mL extract was treated with 1.25 mL Folin–Ciocalteu reagent (Sigma-Aldrich Chemie GmbH, München, Germany) diluted 1:10 with distilled water. The sample was incubated for 5 min at room temperature, and 1 mL Na_2CO_3 60 g/L aqueous salt (Geyer GmbH, Renningen, Germany) was added. The samples were incubated 30 min at 50 °C (INB500 thermostat, Memmert GmbH, Schwabach, Germany) and the absorbance was read at 750 nm (Specord 205; Analytik Jena AG, Jena, Germany). As a blank, 60% ethanol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used. The calibration curve was obtained using gallic acid (concentration range: 2.5–250 µg/mL). The results were expressed in mg gallic acid equivalent (GAE) per g sample, and subsequently transformed in mg GAE per 100 g bilberries fresh weight (mg GAE/100 g FW). All determinations were performed in triplicate.

2.4. Assessment of Antioxidant Activity

The antioxidant activity of the extracts was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay [41]. Briefly, at 0.5 mL extract and 0.5 mL 0.004% (*w*/*v*) solution of DPPH was added. The concentration of investigated extracts where: 10, 5, 2.5, 1.25 and 0.67 mg/mL. The obtained mixture was vortexed, incubated for 30 min at room temperature in dark place, and then the absorbance was read using a spectrophotometer (Specord 205; Analytik Jena AG, Jena, Germany) at 517 nm. Ascorbic

acid was used as positive control. Measurements were taken in triplicate. DPPH radical scavenging activity (RSA) was calculated using the following equation:

$$RSA\ (\%) = (1 - A/A_0) \times 100$$

where A_0 is the absorbance of the negative control, and A is the absorbance in the presence of extract. The negative control was the solution of DPPH.

The half maximal inhibitory concentration (IC_{50}), representing the concentration required to inhibit 50% of free radical, was calculated using a logistic regression based of RSA (%) and corresponding concentrations values. Thus, lower IC_{50} value indicate higher antioxidant activity. The IC_{50} for ascorbic acid was determined to be 0.017 mg/mL.

2.5. Statistical Analyses

The data were statistically processed by combining the analysis of variance (ANOVA) with the additive main effects and multiplicative interaction (AMMI) using MATMODEL Version 3. The significance of differences was analyzed using Multiple Range Test at $p = 0.05$. Data were presented as mean ± SE (standard error). The correlation coefficients were calculated using Pearson method, and the significance was analyzed by a two-tailed test.

The population–year interaction for the studied traits was analyzed using AMMI stability value (ASV), as previously described by Purchase et al. [42]. This value represents the distance to the origin of each population in a two-dimensional space based on coordinates of interaction principal component axis 1–2 (IPCA1–2), considering that lower ASV indicates higher stability.

Broad-sense heritability (h_{bs}^2) was estimated using variance components [43] and was calculated with formula:

$$h_{bs}^2 = V_G/[V_G + (V_{GY}/y) + (V_E/ry)]$$

where: V_G—Genotypic variance; V_{GY}—Variance of genotype–year interaction; V_E—Residual variance; y—number of years (3); r—number of replications (3).

In order to integrate the antioxidant activity data based on radical scavenging activity and IC_{50} into a single value, the relative antioxidant capacity index (RACI) was used. This index described by Sun and Tanumihardjo [44], expresses the mean of standard scores transformed from the initial data.

3. Results

3.1. Total Polyphenol Content

The combined analysis of variance based on the AMMI 2 model for the total polyphenol content (TPC) in the fruits of bilberry populations during the three years, indicates that all three sources significantly influenced this trait, with a predominant action of population and year–population interaction (Table 2). Thus, the year–population interaction showed the highest influence on the variability of TPC (58.59%), followed by the population with a contribution of 34.39%, amid the background of lower influence from the climatic conditions during the study (7.02%).

Cumulated, the six populations achieved yearly average values of TPC between 315.20 mg GAE/100 g FW in 2020 and 432.40 mg GAE/100 g FW in 2018, under a middle variability between years (Table 3). In 2018, the values were significantly higher with 9.77–37.17% compared to those of 2019–2020, with 25% higher accumulation of polyphenols in 2019 compared to 2020.

Regarding the unilateral effect of the bilberry population on TPC, average values from 282.3 mg GAE/100 g FW in the Semenic population to 553.3 mg GAE/100 g FW for the Padis population were registered, amid high inter-population variability. Given the comparisons among the six bilberry populations, it was found that during this period, the Padis population had significantly higher amounts of polyphenols than the other populations, associated with significant increases of 12.17–109.82%. The Cuntu population

also stood out, with 32.04–87.09% significantly higher TPC than four other populations. The Muntele Mic, Semenic, and Sadova Noua populations had close TPC values, and were statistically lower compared to the Raul Lung population.

Table 2. Combined analysis of variance according to the AMMI 2 model for total polyphenol content in bilberry populations during 2018–2020.

Source of Variation	DF	MS	F Value	SS% [1]
Year	2	64275	181.02 **	7.02
Population	5	12599	354.84 **	34.39
Population × Year	10	107314	302.23 **	58.59 (100)
IPCA 1	6	152205	448.16 **	85.10
IPCA 2	4	39976	117.71 **	14.90
IPCA residuals	0			
Residuals	36	355		

DF—Degrees of freedom; MS—Mean square. IPCA—Interaction principal component axes; [1]—% of model sum of squares for year, population, and year–population; ** Significant at $p \leq 0.01$.

Table 3. Total polyphenol content (mg GAE/100 g FW) in bilberry populations during 2018–2020.

Population	Year			Population
	2018	2019	2020	Mean
Muntele Mic	266.50 ± 4.23 d ^{y}	474.90 ± 6.61 c ^{x}	208.80 ± 5.69 c ^{z}	316.70 ± 40.79 D
Raul Lung	385.60 ± 6.33 c ^{y}	155.60 ± 7.83 d ^{z}	579.50 ± 4.82 a ^{x}	373.60 ± 61.83 C
Semenic	482.30 ± 2.01 b ^{x}	144.50 ± 2.22 d ^{z}	220.00 ± 1.38 c ^{y}	282.30 ± 51.22 DE
Cuntu	513.90 ± 4.63 ab ^{y}	654.70 ± 1.63 b ^{x}	311.40 ± 1.94 b ^{z}	493.30 ± 50.07 B
Sadova Noua	403.30 ± 5.63 c ^{x}	137.30 ± 1.65 d ^{z}	250.50 ± 2.11 c ^{y}	263.70 ± 38.73 E
Padis	542.70 ± 3.19 a ^{y}	796.30 ± 4.10 a ^{x}	320.80 ± 1.87 b ^{z}	553.30 ± 68.96 A
Year mean	432.40 ± 22.85 X	393.90 ± 64.44 Y	315.20 ± 30.54 Z	380.50 ± 25.39

Years LSD5% = 20.93; Populations LSD5% = 31.69; Years–population LSD5% = 53.54. Data represents mean ± SE. Different letters (a, b) in the columns indicate significant differences ($p \leq 0.05$) between populations. Different superscript letters ($^{x, y, z}$) in the row indicate significant differences ($p \leq 0.05$) between years. Capital letters were used for year mean (X, Y, Z) and population mean (A, B, C, D, E) comparisons.

Amid the influence of conditions during 2018–2020 on the TPC in each population, the highest amplitude (475.5 3 mg GAE/100 g FW) was registered in the Padis population, while in the case of the Muntele Mic and Sadova Noua populations, the amplitude was considerably lower.

In 2019, the Muntele Mic, Cuntu, and Padis populations achieved significantly higher TPCs compared to the values for 2018 and 2020, statistically differentiated in favor of those from 2018. The Semenic and Sadova populations recorded significantly higher values in 2018 compared to 2020, higher in turn toward the values from 2019. A special reaction was observed in the case of the Raul Lung population, given that in 2020, it accumulated an amount of TPC significantly higher than in 2018–2019, as opposed to lower values in 2019.

Given the conditions of 2018, a lower inter-population variability of TPC was observed, considering that the populations recorded lower amplitudes, ranging from 266.5 mg GAE/100 g FW in Muntele Mic and 542.7 mg GAE/100 g FW in Padis. The highest values of TPC in 2018 were recorded in the Padis population, which showed significant increases of 2.52–103.6% to most other populations. In the case of the Cuntu and Semenic populations, the TPC was significantly higher than in Sadova Noua and Raul Lung populations, which were statistically undifferentiated.

Regarding the effect of the conditions of 2019 on TPC, it was observed that the populations recorded an amplitude of 659 mg GAE/100 g FW, considerably higher than other years, with values between 144.5 mg GAE/100 g FW in Semenic and 796.3 mg GAE/100 g FW in Padis, amid a very high inter-population variability. Thus, the largest amount of polyphenols was accumulated by the fruit of the Padiş population, associated with significant increases of over 21.63% compared to other populations. In the case of the Cuntu population, the TPC values were significantly higher by 179.8–510.2% compared

to the other four populations. In this year, the Semenic, Raul Lung, and Sadova Noua populations accumulated significantly lower amounts of polyphenols in their fruits.

Given the conditions from 2020, the bilberry populations had TPC values ranging from 208.8 mg GAE/100 g FW in Muntele Mic and 579.5 mg GAE/100 g FW in Raul Lung, with an amplitude of 370.7 mg GAE/100 g FW and a high inter-population variability. Thus, the Raul Lung population showed a significantly higher TPC, over 80.64% greater than the other populations. In the case of the Cuntu and Padis populations, the values were 24.31–53.64% significantly higher compared to the Sadova Noua, Semenic, and Muntele Mic populations, which were statistically undifferentiated.

Depending on the values of the ASV index, it is noted that the Semenic, Sadova Noua, and Muntele Mic populations had the highest TPC stability, on a background of a qualitative (different signs of IPCA) type of population–year interaction (Table 4). In the case of the Padis and Raul Lung populations, the amounts of polyphenols displayed large deviations during the study, associated with a qualitative interaction in the Padis population and a quantitative interaction in the Raul Lung population. In this regard, the low stability of the Raul Lung population was associated with a TPC value below the overall mean, while in the Padis population, the low stability of TPC was correlated with values above the mean.

Taking into account the fact that IPCA1 displays a considerable contribution (85.1%) to total variability, depending on the position in the biplot from Figure 1, it was noted that the Raul Lung and Padis populations had the lowest stability of TPC during the study. Based on the length of the associated vectors, it was found that the conditions of 2019–2020 had high contributions to the population–year interaction for this trait. Depending on the position towards the year vectors, it was observed that the Raul Lung population had a specific adaptation to the conditions of 2020, while the Semenic population efficiently used the conditions of 2018. In the case of the Padis and Cuntu populations, specific adaptations to the conditions of 2019 were observed.

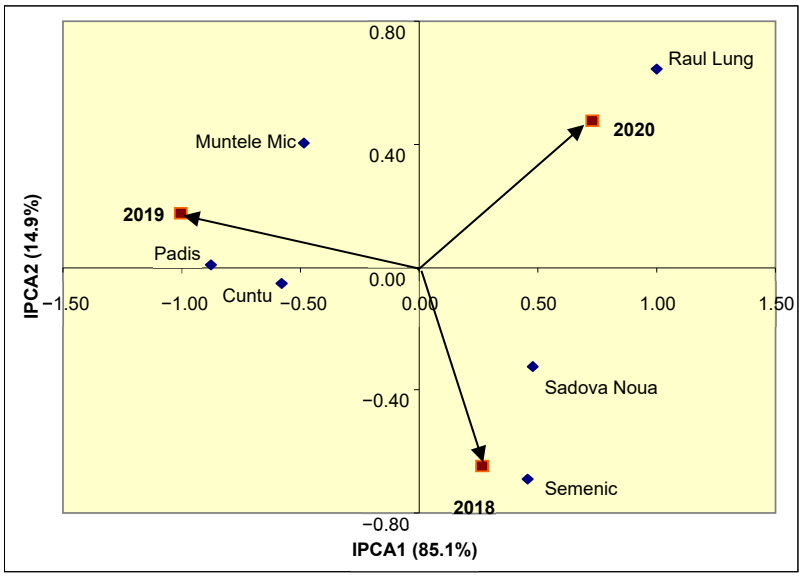

Figure 1. Biplot of interaction for principal component axis (IPCA1 and IPCA2) for total polyphenol content in bilberry populations during 2018–2020.

Table 4. Analysis of population–year interaction for total polyphenol content in bilberry using the AMMI stability value (ASV).

Population	IPCA1	IPCA2	ASV	
			Value	Rank
Muntele Mic	−0.484	0.406	2.793	3
Raul Lung	1.000	0.646	5.747	6
Semenic	0.459	−0.689	2.711	1
Cuntu	−0.576	−0.051	3.291	4
Sadova Noua	0.480	−0.322	2.757	2
Padis	−0.879	0.009	5.018	5

IPCA—Interaction principal component axes.

3.2. DPPH Radical Scavenging Activity

For DPPH radical scavenging activity, the results of combined analysis of variance according to the AMMI 2 model, indicates that all three main sources of variation have a significant influence (Table 5). The contributions of variation sources are differentiated, thus the variability of this indicator was more influenced by the conditions during the three years (63.51%), while the effects of population (14.58%) and year–population interaction (21.90%) were considerably lower. Given that the first two IPCA fully express the effect of population–year interaction, it follows that the AMMI2 model is suitable for assessing the antioxidant activity of fruit extracts in these six populations.

Table 5. Combined analysis of variance according to the AMMI 2 model for DPPH radical scavenging activity of fruit extracts in bilberry populations during 2018–2020.

Source of Variation	DF	MS	F Value	SS% [1]
Year	2	822.11	28.71 **	63.51
Population	5	75.50	2.64 *	14.58
Population × Year	10	56.70	1.98 *	21.90 (100)
IPCA 1	6	78.19	27.22 **	82.74
IPCA 2	4	24.47	8.52 **	17.26
IPCA residuals	0			
Residuals	36	28.63		

DF—Degrees of freedom; MS—Mean square. IPCA—Interaction principal component axes; [1]—% of model sum of squares for year, population, and year–population; * Significant at $p \leq 0.05$; ** Significant at $p \leq 0.01$.

Considering the cumulative effect of the years, during this period (Table 6), average values of inhibition ranging from 72.09% in 2020 to 81.94% in 2018 were found, with low variation between the three years. Generally, the bilberry populations recorded during 2018–2019 had significant increases of radical scavenging activity, equivalent to values about 8–9% higher than the results of 2020. Additionally, the conditions of 2018 favored the manifestation of a significantly higher inhibition compared to the conditions of 2019.

The average values of radical scavenging activity for the six populations had an amplitude of 5.83%, with limits from 75.58% for the Cuntu population, up to 81.41% for the Padiş population, with low inter-population variability. Compared to the overall mean, the Padis and Raul Lung populations recorded significant increases of 1.97–3.37%, while for other populations, the inhibition was significantly lower than the mean. Based on comparisons between populations, it was found that the Padis population had a radical scavenging activity over 1.40% significantly higher than the other populations, followed by the Raul Lung population, which recorded significant deviations of 2.38–4.43% compared to the rest of the statistically differentiated populations.

Given the population–year interaction, it was observed that under the conditions of 2018, the inhibition recorded values between 78.90% in the Cuntu and 85.72% in the Padis populations, with an amplitude of 6.82% and low variability between populations. Given this year's conditions, the Padis population had a radical scavenging activity 3.51%

significantly greater than the other populations. Additionally, the Muntele Mic, Sadova Noua, and Semenic populations registered inhibitions 1.55–3.31% higher than the Raul Lung and Cuntu populations.

Table 6. DPPH radical scavenging activity (%) of fruit extracts for bilberry populations during 2018–2020.

Population	Year			Population
	2018	2019	2020	Mean
Muntele Mic	82.11 ± 1.60 b ^{x}	81.78 ± 1.27 a ^{x}	69.01 ± 1.28 c ^{y}	77.63 ± 1.21 C
Raul Lung	80.61 ± 1.32 c ^{y}	81.24 ± 1.32 ab ^{x}	78.16 ± 1.16 b ^{z}	80.01 ± 0.74 B
Semenic	82.06 ± 0.78 b ^{x}	80.80 ± 1.29 b ^{y}	69.34 ± 1.06 c ^{z}	77.40 ± 1.05 C
Cuntu	78.90 ± 1.90 d ^{y}	79.64 ± 1.27 c ^{x}	68.19 ± 0.91 d ^{z}	75.58 ± 0.98 E
Sadova Noua	82.21 ± 1.07 b ^{x}	78.78 ± 1.27 d ^{y}	67.67 ± 1.32 e ^{z}	76.22 ± 1.16 D
Padis	85.72 ± 1.94 a ^{x}	78.34 ± 1.28 d ^{z}	80.17 ± 1.49 a ^{y}	81.41 ± 1.01 A
Year mean	81.94 ± 0.57 X	80.10 ± 0.53 Y	72.09 ± 0.72 Z	78.04 ± 0.44

Years LSD5% = 0.74; Populations LSD5% = 0.26; Year–population LSD5% = 0.60. Data represents mean ± SE. Different letters (a, b, c, d, e) in the column indicate significant differences ($p \leq 0.05$) between populations. Different superscript letters ($^{x, y, z}$) in the row indicate significant differences ($p \leq 0.05$) between years. Capital letters were used for year mean (X, Y, Z) and population mean (A, B, C, D, E) comparisons.

Regarding the radical scavenging activity in 2019, amid an inter-population variability close to the previous year, values between 78.34% in Padis and 81.78% in Muntele Mic were recorded, with an amplitude of 3.44% and a grouping of populations in five classes. Thus, the Muntele Mic population used this year's conditions at a higher level, showing significantly higher inhibition of over 0.54% compared to the other populations. In the case of the Sadova Noua and Padis populations, close values were observed, associated with significantly lower radical scavenging activity than the Cuntu, Semenic, and Raul Lung populations.

Given the conditions of 2020, the populations registered a high amplitude of 12.50%, ranging between 67.67% in Sadova Noua and 80.17% in Padis. As such, this year the Padis population showed the highest radical scavenging activity, associated with significant increases of over 2.01% compared to other populations. Additionally, the Raul Lung population recorded a significantly higher inhibition of 9.15–10.39% compared to four other populations. The Muntele Mic and Semenic populations had similar antioxidant activities amid an inhibition 1.15–1.67% higher than the Cuntu and Sadova Noua populations.

Considering the effect of the variability in yearly conditions during the study on the radical scavenging activity in each population, it was observed that the Semenic and Sadova Noua populations capitalized the conditions of 2018 at a higher level, registering a significantly higher inhibition compared to the statistically differentiated values of 2019–2020. In the period 2018–2019, the Muntele Mic and Raul Lung populations presented close and significantly higher values of inhibition by 3.08–13.1% compared to 2020. The fruits of the Cuntu population showed significantly higher radical scavenging by 0.74–11.45% in 2019 compared to the values of 2018 and 2020, respectively. Additionally, in the case of the Padis population, the conditions from 2018 favored a significantly higher expression of inhibition compared to the statistically differentiated values from 2019–2020.

Regarding the unilateral effect of extract concentration (Table S1), the inhibition showed an amplitude of 12.90%, with mean values ranging from 71.66% for 0.67 mg/mL concentration to 84.57% in the case of the maximum concentration. Thus, at the level of the whole study, there is a significant reduction of the radical scavenging activity proportional to the decrease of extract concentration. The rate of inhibition decrease was between 0.67%/mg/mL when the concentration was changed from 10 to 5 mg/mL, and 5.43%/mg/mL when the concentration was reduced from 1.25 to 0.67 mg/mL, respectively.

Regarding the combined effect of the extract concentration and the population on the radical scavenging activity, it is observed that the fruits of the Padis population showed the highest antioxidant activity regardless of extract concentration. For the 10 mg/mL

concentration, inhibition registered values between 81.09% in Cuntu and 89.22% in Padis populations, amid significant deviations among most of the populations except for Semenic and Sadova Noua, which showed close values. Under the effect of 5 mg/mL concentration, the radical scavenging activity varied from 78.54% in Cuntu to 85.62% in Padis populations, amid higher values of 81.4–83.08% in Muntele Mic and Raul Lung populations and significantly lower values of 78.73–79.89% in Sadova Noua and Semenic populations. The extracts with a concentration of 2.5 mg/mL showed inhibitions from 75.07% in Cuntu to 81.31% in Padis, associated with a 6.24% amplitude and significant differentiation between populations.

For the 1.25 mg/mL concentration, inhibition registered values between 72.74% in Cuntu and 77.81% in Padiş, with significant deviations among most of the populations, with the exception of Cuntu and Sadova Noua, which recorded close values. Under the effect of 0.67 mg/mL concentration, the radical scavenging activity varied from 70.46% in Cuntu to 73.51% in Raul Lung, associated with values of 71.52–73.09% in Semenic and Padiş, and significantly lower values of 70.47–70.94% in the Sadova Noua and Muntele Mic populations. Thus, the antioxidant activity of the extracts of the Cuntu, Sadova Noua, and Muntele Mic populations at a concentration of 0.67 mg/mL were equivalent to the antioxidant activity of ascorbic acid at a concentration of 0.08 mg/mL (Table S2). In the case of Padis and Raul Lung populations, during the period of study the antioxidant activity at the concentration of 0.67mg/mL was higher than that of ascorbic acid at a concentration of 0.1 mg/mL.

Based on ASV ranks (Table 7), it was observed that the highest stability of radical scavenging activity manifested in the Cuntu population on a background of a qualitative population–year interaction, and a value below the overall mean. In the case of the Raul Lung and Padis populations, antioxidant activity registered considerable deviations during the study in the presence of mixed interactions at Padis and a quantitative one at the Raul Lung population, respectively. The Semenic and Sadova Noua populations had a cross interaction, registering values of antioxidant activity in accordance with the variation of the year's mean.

Table 7. Analysis of population–year interaction for DPPH radical scavenging activity of fruit extracts in bilberry using the AMMI stability value (ASV).

Population	IPCA1	IPCA2	ASV	
			Value	Rank
Muntele Mic	−0.522	0.048	2.504	4
Raul Lung	0.675	0.664	3.303	5
Semenic	−0.397	−0.056	1.905	2
Cuntu	−0.328	0.298	1.602	1
Sadova Noua	−0.427	−0.424	2.090	3
Padis	1.000	−0.529	4.821	6

IPCA—Interaction principal component axes.

Given that IPCA1 expresses approximately 83% of the variability of the population–year interaction (Figure 2), and depending on the coordinates of each year, it was found that the conditions of 2020 had a higher contribution to this interaction, while the conditions of 2018 had a lesser influence on the variation of radical scavenging activity in the six bilberry populations.

Depending on the distance from the origin, it was observed that the antioxidant activity of the Padis and Raul Lung populations was strongly influenced by the population–year interaction, while in the Cuntu and Semenic populations, this trait had greater stability. The close position of the Cuntu population towards the 2019 vector indicates a specific adaptation to this year's conditions, registering the highest value of this trait, while in the Sadova Noua population, the conditions from 2018 favored superior radical scavenging activity.

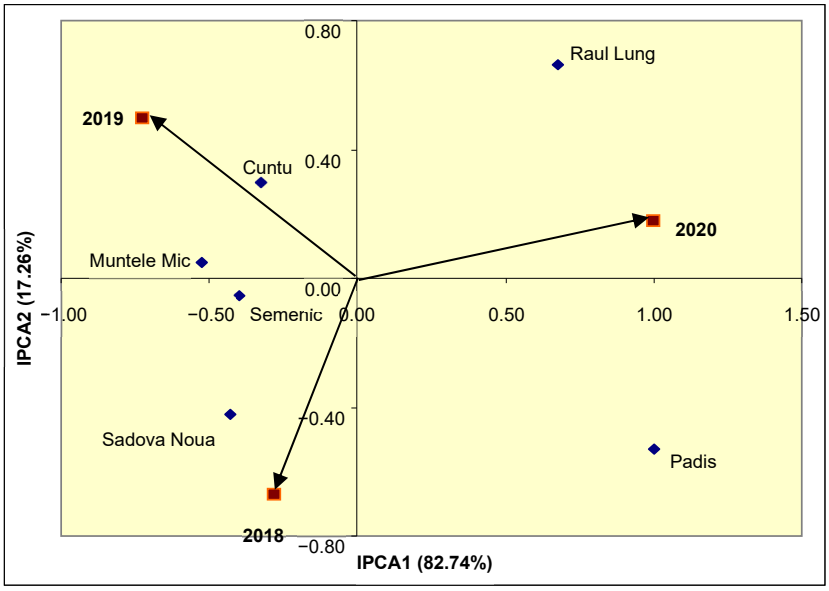

Figure 2. Biplot of interaction for principal component axis (IPCA1 and IPCA2) for DPPH radical scavenging activity of fruit extracts in bilberry populations during 2018–2020.

3.3. Half Maximal Inhibitory Concentration (IC_{50})

The combined analysis of variance based on the AMMI 2 model for the half maximal inhibitory concentrations (IC_{50}) of extracts for bilberry populations during three years, indicates that all three sources significantly influenced this trait, with predominant action from population and a lower contribution from year (Table 8). Thus, population had the highest influence on the variability of IC_{50} values (51.86%), followed by the population–year interaction with a contribution of 34.36%, on a background of lower influence of year conditions during the study (13.96%).

Table 8. Combined analysis of variance according to the AMMI 2 model for half maximal inhibitory concentration (IC_{50}) of fruit extracts from bilberry populations during 2018–2020.

Source of Variation	DF	MS	F Value	SS% [1]
Year	2	0.0004	11.75 **	13.96
Population	5	0.0006	17.40 **	51.68
Population × Year	10	0.0002	5.79 **	34.36 (100)
IPCA 1	6	0.0003	9.09 **	91.77
IPCA 2	4	0.00004	1.21 [ns]	8.23
IPCA residuals	0	0.00003	11.75	
Residuals	36			

DF—Degrees of freedom; MS-Mean square. IPCA—Interaction principal component axes; [1]—% of model sum of squares for year, population and year–population; ** significant at $p \leq 0.01$; [ns]—non-significant.

Overall, during the study, the half maximal inhibitory concentration (IC_{50}) had low variability, associated with an amplitude of 0.009 mg/mL, ranging from 0.04 mg/mL in 2020 to 0.049 mg/mL in 2019 (Table 9). The conditions of 2020 had a positive influence on the antioxidant activity evaluated through IC_{50}, registering significant increases compared to the results of 2018–2019. Additionally, under the conditions of 2018–2019, the antioxidant activity based on IC_{50} registered close values.

In this period, the Muntele Mic and Semenic populations had antioxidant activity significantly higher than the other populations except for Raul Lung, achieving the lowest average values (0.038–0.039 mg/mL) of IC_{50}. The Sadova Noua and Cuntu populations recorded the lowest antioxidant activities, associated with high average values (0.049–0.059 mg/mL) of IC_{50}.

Considering the combined effect of years and populations on the IC_{50}, it was observed that given the conditions of 2018, the populations registered an amplitude of 0.024 mg/mL, with values between 0.037 mg/mL in Muntele Mic and 0.061 mg/mL in Sadova Noua. The highest antioxidant activity was seen in the Muntele Mic, Raul Lung, and Padis populations, being associated with significantly lower IC_{50} values than the other populations.

Table 9. Half maximal inhibitory concentration (IC_{50}) of fruit extracts of bilberry populations during 2018–2020.

Population	Year			Population
	2018	2019	2020	Mean
Muntele Mic	0.037 ± 0.004 c ^{x}	0.039 ± 0.003 c ^{x}	0.038 ± 0.005 bc ^{x}	0.038 ± 0.002 D
Raul Lung	0.040 ± 0.001 c ^{x}	0.040 ± 0.001 c ^{x}	0.044 ± 0.002 ab ^{x}	0.041 ± 0.001 DC
Semenic	0.045 ± 0.003 bc ^{x}	0.037 ± 0.001 c ^{xy}	0.030 ± 0.002 c ^{y}	0.039 ± 0.002 D
Cuntu	0.053 ± 0.001 ab ^{x}	0.058 ± 0.006 b ^{x}	0.038 ± 0.001 bc ^{y}	0.049 ± 0.003 B
Sadova Noua	0.061 ± 0.003 a ^{y}	0.076 ± 0.008 a ^{x}	0.042 ± 0.001 ab ^{z}	0.059 ± 0.005 A
Padis	0.043 ± 0.004 c ^{xy}	0.037 ± 0.004 c ^{y}	0.049 ± 0.001 a ^{x}	0.045 ± 0.002 BC
Year mean	0.046 ± 0.002 X	0.049 ± 0.004 X	0.040 ± 0.002 Y	

Years LSD5% = 0.004; Populations LSD5% = 0.006; Year x population LSD5% = 0.010. Data represents mean ± SE mg/mL. Different letters in the column (a, b, c) indicate significant differences ($p \leq 0.05$) between populations. Different superscript letters ($^{x, y, z}$) in the row indicate significant differences ($p \leq 0.05$) between years. Capital letters were used for year mean (X, Y) and population mean (A, B, C, D) comparisons.

Amid the conditions of 2019, the bilberry populations recorded IC_{50} values from 0.037 mg/mL in Semenic and Padis to 0.076 mg/mL in Sadova Noua, with an amplitude of 0.039 mg/mL, higher than the previous year. This year, the Semenic, Padis, Muntele Mic, and Raul Lung populations showed the highest antioxidant activity, associated with significantly lower IC_{50} values than the Sadova Noua and Cuntu populations.

In the conditions of 2020, the IC_{50} value had lower variability between populations than in previous years, with values from 0.03 mg/mL in Semenic to 0.049 mg/mL in Padis, on a background of lower antioxidant activity compared to the results of 2018–2019. The Semenic population had a higher antioxidant activity and a significantly lower IC_{50} than the Padis, Raul Lung, and Sadova Noua populations.

In the case of the Muntele Mic and Raul Lung populations, the IC_{50} values had small and no significant variations during the study. In the Sadova Noua population, significant variations of antioxidant activity from one year to another were observed, associated with higher IC_{50} values in 2019 and lower in 2020. The Semenic and Cuntu populations registered significantly higher antioxidant activities in 2020 than in 2018, while in the Padis population, the IC_{50} value in 2019 was significantly lower than that of 2020.

Depending on the values of the ASV index (Table 10), it was noted that the Semenic and Muntele Mic populations had the highest stability of IC_{50}, on a background of a quantitative (same signs of IPCA) type of population–year interaction. In the case of the Sadova Noua population, the IC_{50} value recorded large deviations during the study, associated with a qualitative interaction.

Considering that IPCA1 has a considerable contribution (91.77%) to total variability, based on the position in the biplot from Figure 3, it was noted that the Semenic and Muntele Mic populations expressed the highest stability of IC_{50} during the study. Based on the year's associated vectors, it was found that the conditions from 2018–2019 had close contributions to the population–year interaction. Depending on the position towards the year vectors, it was observed that the Sadova Noua population had the lowest stability of IC_{50} amid specific adaptation to the conditions of 2019. In the case of the Padis and Raul Lung populations, specific adaptations to the conditions of 2020 were observed.

Based on the relative antioxidant capacity index (RACI) value of the six bilberry populations (Figure 4), it was noted that the Padis and Raul Lung populations showed the highest antioxidant activity, while the Sadova Noua and Cuntu populations had significantly lower values of this index and relatively week antioxidant activity, respectively.

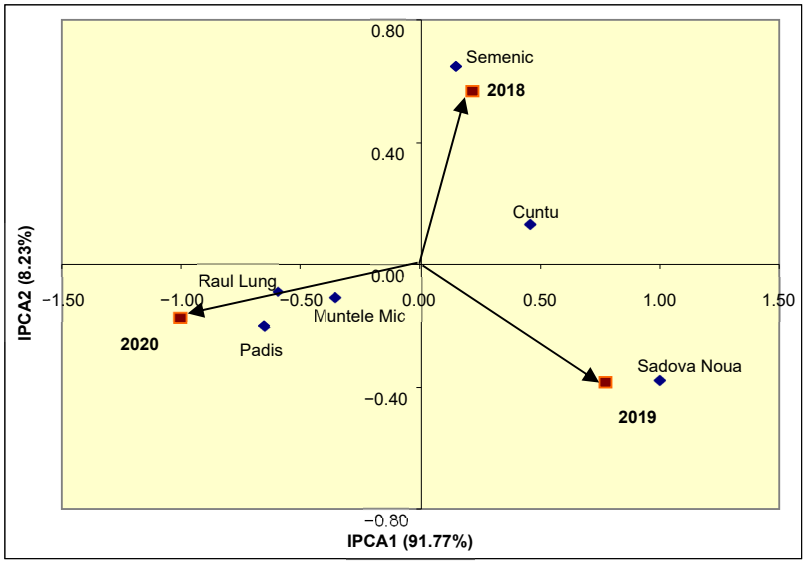

Figure 3. Biplot of interaction for half maximal inhibitory concentration (IC_{50}) of fruit extracts of bilberry populations during 2018–2020.

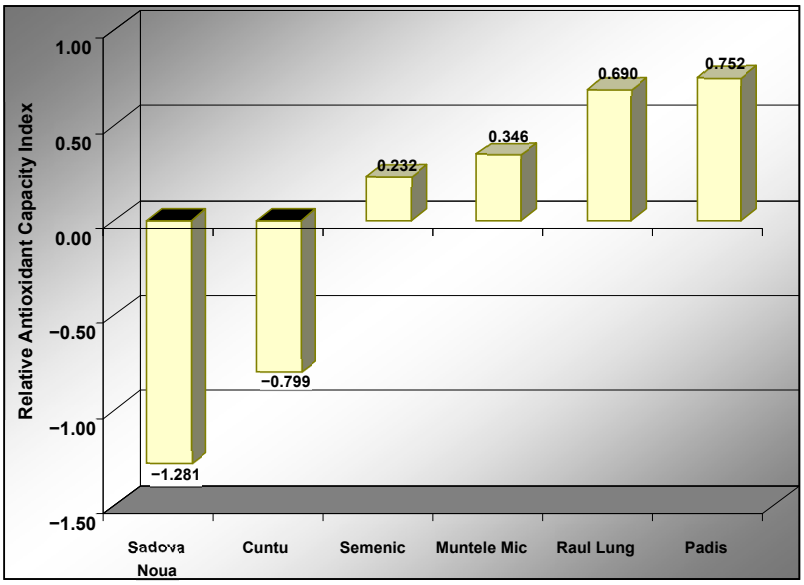

Figure 4. Relative antioxidant capacity index of bilberry populations.

Table 10. Analysis of population–year interaction for half maximal inhibitory concentration (IC_{50}) of fruit extracts of bilberry using the AMMI stability value (ASV).

Population	IPCA1	IPCA2	ASV	
			Value	Rank
Muntele Mic	−0.356	−0.108	3.971	2
Raul Lung	−0.597	−0.089	6.657	4
Semenic	0.147	0.647	1.762	1
Cuntu	0.459	0.133	5.120	3
Sadova Noua	1.000	−0.380	11.156	6
Padis	−0.653	−0.203	7.284	5

IPCA—Interaction principal component axes.

3.4. Variance Components and Correlations

In the case of polyphenol content (Table 11), major differences were observed among phenotypic and genotypic variances, and among the two coefficients of variation, respectively. This fact indicates that the phenotypic expression of this trait has been considerably influenced by the population–year interaction. The low value of broad sense heritability (14.83%), associated with the fact that the genotypic variance was significantly lower than other variances, suggests that in these six bilberry populations, the polyphenol content is mainly controlled by environmental conditions and non-additive genes.

Table 11. Variance components, broad sense heritability, and coefficients of variation for analyzed traits in bilberry populations during 2018–2020.

Traits	V_G	V_{GY}	V_P	h^2_{bs} (%)	GCV	PCV
Total polyphenols content	2.076	35.651	13.999	14.83	11.97	31.10
DPPH radical scavenging activity	1.253	10.837	5.033	24.89	1.43	2.87
Half-inhibitory concentration (IC_{50})	0.00007	0.0001	0.0001	66.75	11.68	9.54

V_G—Genotypic variance; V_P—Phenotypic variance; V_{GY}—Variance of genotype–year interaction; h^2_{bs}—Broad sense heritability; GCV—Genotypic coefficient of variation; PCV—Phenotypic coefficient of variation.

In the case of radical scavenging activity on the background of variation coefficients that attest a very low variability, it was noted that genotypic variance has a low contribution to phenotypic variance, having at the same time a significantly lower value compared to the interaction variance. Considering the low value of broad sense heritability (24.89%), it turns out that this trait is strongly influenced by non-additive genes in interaction with environmental conditions.

In the case of IC_{50} on the background of close values for the coefficients of variation, it was noted that phenotypic variance has a similar contribution as the interaction variance to the total variability. Considering the heritability value (56.50%) as well, it can be stated that in the inheritance of this trait, genes with additive effect also act.

Considering the correlations between the analyzed traits of bilberry fruits and environmental characteristics, it was found that the TPC had a positive relationship with soil pH and phosphorus content, and weak correlations with soil nitrogen and potassium contents (Table 12). The DPPH radical scavenging activity had a moderate positive correlation with pH and phosphorus content, and negative correlations with nitrogen and potassium contents in soil. IC_{50} values are significantly negatively correlated with altitude and positively correlated with potassium content, suggesting that the inhibitory concentration of fruit extracts decreases with increasing altitude and decreasing potassium content.

Table 12. Pearson correlations between polyphenol content and antioxidant activity of bilberry fruits with environmental characteristics.

	Altitude(m)	pH	N (%)	P (ppm)	K (ppm)
Total polyphenol content	0.345	0.687	−0.038	0.791	−0.108
DPPH radical scavenging activity	0.171	0.478	−0.353	0.354	−0.405
Half inhibitory concentration (IC_{50})	−0.855 *	0.240	0.609	0.438	0.871 *

* Significant at $p \leq 0.05$.

4. Discussion

The content of secondary metabolites in different plant organs varies due to modifications of gene expression or of their encoded protein activity involved in secondary metabolism under the effects of different developmental stages and stresses [45]. The synthesis of phenolic compounds is a combinatorial biosynthesis, including the pathways of shikimate and acetate [46]. Further, in bilberry, genotype can significantly affect the amount and qualitative composition of different phenolic compounds in fruits [47]. Variations in polyphenolic compositions were highlighted in bilberry populations from various

harvesting regions, probably as a consequence of the cumulative effects of genotype and environment [48].

Given the range of TPC in the fruits of many species [49], in our research most bilberry populations had a medium content of 100–500 mg GAE/100 g FW, except for the Padis population, whose TPC was higher. These results are close to TPC values determined in bilberries from other regions of Romania, such as: 331–419 mg GAE/100 g FW in Borca [50] and 355 mg GAE/100 g FW in Breaza [51]. Furthermore, the values of TPC in the six studied bilberry population were lower compared to those of 672–819 mg GAE/100 g FW recorded in bilberries from northwest Romania [31].

The maximum values of TPC recorded in our study for each population are comparable to those reported in bilberries from other European countries: 577–614 mg GAE/100 g FW in Appennino Modenese, Italy [32]; 531–674 mg GAE/100 g FW in Norway [52]; 498–563 mg GAE/100 g FW in Coruh, Turkey [29], 640 mg GAE/100 g FW in Sulechov, Poland [53]. At the same time, higher values of TPC were reported for bilberry populations from: Fojnica, Bosnia (803–1040 mg/100 g FW) [54]; Velingrad-Troyan, Bulgaria (893–940 803–1040 mg/100 g FW) [55]; or Kopaonik, Serbia (808 mg GAE/100 g FW) [56].

The mean TPC values in our bilberry populations fell within the reported results for bilberries from other countries and regions, such as: 403 mg GAE/100 g FW in Borja [57] and 431–455 mg GAE/100 g FW in Bugojno, Bosnia and Herzegovina [58]; 376–476 mg GAE/100 g FW in Rakitovo-Yundola, Bulgaria [59]; 392–524 in northeast Montenegro [17]; 366–457 mg GAE/100g FW in Mazovia, Polonia [60]; 387 mg GAE/100 g FW in Dragacevo, Serbia [30]; 367–442 mg GAE/100 g FW in Velka Fatra and Tatra, Slovakia [16]. During the study, the yearly values of TPC for most bilberry populations were higher compared to the results of 200–215 mg GAE/100 g FW obtained in bilberries from northeast Turkey [61].

The antioxidant activity based on the radical scavenging activity (RSA) for the six bilberry populations range from 75.58% in Cuntu to 81.41% in Padis, and was higher compared to those of 50–60% associated with bilberry populations from northeast Romania, or 30–46% for cultivated blueberries in the same region [31]. Additionally, lower values of RSA estimated through the same DDPH method were reported in other studies: 28%, in bilberries from Japan [62]; 34%, in bilberries from Canada [35]: 43%, in bilberries from Slovenia [63]; and 62%, in bilberries from South Korea [28].

In our study, the average IC_{50} (concentration required to inhibit 50% of free radical) varied from 0.038–0.039 mg/mL (the highest antioxidant activity) for the Muntele Mic population to 0.059 mg/mL (the lowest antioxidant activity) for the Sadova Noua population. Our results are in agreement with the findings of Guder et al. [64], who reported an IC_{50} value of 0.0275 mg/mL in bilberries from Turkey, which also showed very effective inhibitory activity against α-amylase glucosidase, with an IC_{50} value of 0.0655 mg/mL. The bilberry populations from our study showed the strongest antioxidant activity, with lower IC_{50} values compared to the values of 1.8–2 mg/mL obtained by Samad et al. [65] in cultivated blueberry. The antioxidant activity of bilberries from Turkey expressed as IC_{50} values (0.229 mg/mL) was significantly higher compared with cultivated blueberry (IC_{50} = 1.178 mg/mL), as reported by Saral et al. [66]. In a similar study, Stefanescu et al. [23] concluded that the antioxidant activity of bilberry from central Romania (IC_{50} = 0.175 mg/mL) was much higher compared with cultivated *Vaccinium corymbosum* (IC_{50} = 1.47 mg/mL), and that the cold storage of fruits for three months at −20 and −50 °C significantly decreased the antioxidant activity by increasing the IC_{50} from 0.175 mg/mL to 1.03–1.09 mg/mL.

In a study regarding the effects of bilberry on cancer cell lines, Durmaz et al. [67] found an IC_{50} value of 0.0291 mg/mL for caffeic acid, 0.0399 mg/mL for p-coumaric acid, and 0.239 mg/mL for ferulic acid. Choi et al. [68] reported that blueberry weakly inhibited diphospho-glucuronosyltransferase, with an IC_{50} value of 0.0624 mg/mL. Proanthocyanidins from blueberry showed inhibitory activity against prostate cancer cells, with the highest effect of an IC_{50} value of 0.0744 mg/mL [69].

Considering the soil characteristics, our results indicate that the highest TPC was recorded by the Padis and Cuntu populations from sites with the highest values of soil pH and phosphorus content. The lowest TPC values were registered in the Semenic population in soils with low pH and phosphorus content associated with high nitrogen content, and also by the Sadova Noua population, with the lowest altitude and highest potassium and nitrogen content in the soil. The negative effect of nitrogen on antocyanin content and TPC in bilberry fruits was reported by different studies in Norway [52] and Sweden [70], similar to the findings of Eicholz et al. [71], who reported that in blueberry cultivars the TPC and antioxidant activity decreased with higher N fertilization.

The low values of broad sense heritability, associated with the fact that the genotypic variance was significantly lower than the other variances, suggests that for these six bilberry populations, the polyphenol content and antioxidant activity were highly influenced by environmental factors. Studying a large collection of 100 tetraploid blueberry accession, Mengist et al. [72] reported moderate to high broad sense heritability for all anthocyanin metabolites and a low heritability for flavanols, flavonols, and phenolic acids. In research based on blueberry progenies, Connor et al. [73] estimated moderate heritability for antioxidant activity (43%), total phenols (46%), and anthocyanin content (56%). These higher values of heritability in cultivated blueberries compared to our results are probably due to the fact that in the wild, the variation of environmental factors is much higher. The influence of the genotype–environment interaction in the accumulation of phenolic compounds, especially anthocyanins, was also detected in natural bilberry populations from different European countries [17,24,74].

5. Conclusions

This study provided for the first time information concerning the quantity of polyphenols and antioxidant potential of bilberry populations from the Banat Mountains, under specific environmental conditions. The Padis and Raul Lung populations had the highest values of polyphenol content and antioxidant activity, associated with considerable influence of the population–year interaction. During 2018–2019, all populations regardless of extract concentration, showed higher RSA inhibition than maximum related to ascorbic acid. Given the well known health benefits attributed to polyphenols, the fruits of these bilberry populations could be considered potential sources of antioxidants for direct consumption, or use as ingredients for food products or food supplements. The high polyphenol content in the Cuntu population was associated with low antioxidant activity, suggesting that the amount of different phenolic compounds in their fruits can be different compared with other populations.

The low level of broad sense heritability for total polyphenol content and antioxidant activity associated with the high effects of year and population–year interaction indicates that the accumulation of polyphenols in bilberry fruits is governed by complex enzymatic activities, which can react differently depending on changes in environmental conditions. In this regard, significant variation of total polyphenol content and antioxidant activity was observed both between the two regions (Apuseni and Banat Mountains), and among the populations from Banat Mountains. The present study represents a first step in the context of finding new natural solutions for enriching functional foods in active principles. In this sense, further studies could aim to include lyophilized bilberry extracts enriched in polyphenols into premix-type flour foods, biscuits, or pastry/confectionery products, in order to promote a healthy diet.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11122557/s1. Table S1. DPPH radical scavenging activity (%) for different concentrations of fruit extracts in bilberry populations; Table S2. DPPH radical scavenging activity (%) for different concentrations of fruit extracts in bilberry populations during 2018–2020.

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