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Original article – Thematic Issue

Influence of ripeness stage on the rate of pigment degradation in blackberry fruits during frozen storage

Ž. Karaklajić-Stajić[1,*], M. Nikolić[2], J. Tomić[1], R. Miletić[1] and M. Pešaković[1]

[1] Fruit Research Institute Čačak, Čačak, Serbia

[2] Faculty of Agriculture, University of Belgrade, Belgrade, Serbia

Summary

Background of the study – **The high proportion of the blackberry (*Rubus* subg. *Rubus* Watson) cultivar Čačanska Bestrna in commercial orchards (about 60%) in the Republic of Serbia is one of the reasons for the standstill in blackberry marketing as the market value of blackberries is often lowered due to the poor stability of their black colour during frozen storage. With this in mind, the aim of this study was to determine the relationship between the rate of pigment degradation and soluble solids content in the blackberry fruit during frozen storage.** *Materials and methods* – **Blackberry fruits of three cultivars were collected at different ripeness stages and frozen at -20°C for 7 and 30 days. Pigment degradation was determined by counting fruits showing pigment degradation after two freezing periods. Soluble solids content (SSC) was measured by a digital refractometer, and total anthocyanins content (TAC) was assessed by the pH-differential method.** *Results and discussion* – **The highest rate of pigment degradation (PD) during freezing was found in 'Čačanska Bestrna', as induced by the lowest SSC and TAC. A lower rate of PD was recorded in berries sampled at the over-ripe stage, whereas an increase in the rate of PD and a decrease in SSC and TA contents were determined during frozen storage.** *Conclusion* – **To prolong the shelf life of blackberries whilst maintaining the health benefits and stable colour of fruits, freezing is the best method, but the rate of PD, SSC and TAC during freezing varies with cultivar and ripeness stage.**

Keywords

anthocyanins, blackberry, freezing, pigment degradation, soluble solids

Significance of this study

What is already known on this subject?

- The intense black color of blackberries depends on the stability of anthocyanins in freezing conditions and the shelf life can increase by different ripeness stages, frozen storage period, and cultivar.

What are the new findings?

- This study clearly demonstrated that higher SSC in blackberries increased stability of anthocyanins in freezing conditions, i.e., decreased pigment degradation in 'Čačanska Bestrna', 'Loch Ness' and 'Chester Thornless'.

What is the expected impact on horticulture?

- Relationship between the sugar content and anthocyanins of the blackberries can provide guidelines for growers to select appropriate picking time in order to improve quality for off-season use.

Introduction

Small fruits have an exceptional nutritional potential based on their high content of natural antioxidant compounds (Ding et al., 2006; Tulipani et al., 2008) which exhibit anti-inflammatory, antiviral, antimicrobial and antioxidant activities (Reyes-Carmona et al., 2005). A wide range of phenolics occur in different kinds of berries, which are a rich source of anthocyanins, flavonols, flavanols and other flavonoids (Mikulic-Petkovsek et al., 2012; Mitic et al., 2014). These compounds are responsible for the high free radical scavenging capacity measured by in vitro and in vivo assays (Kaume et al., 2012; Slatnar et al., 2012a). Among commercially important small fruits, blackberries stand out for their highest antioxidant capacity (AC) of the fruit (Pantelidis et al., 2007), but Kaume et al. (2012) reported variations in the chemical composition of blackberries depending on cultivar, growing conditions, ripeness stage and other factors.

Fruit attractiveness, i.e., the visual aspect of fruit quality, is determined by shape, colour and size, and plays a key role in fruit acceptability to consumers. As consumers evaluate fruit quality by colour, it is necessary to retain the original fruit colour and ensure product uniformity for increased market acceptance (Giusti and Wrolstad, 2003; Wu et al., 2006). Similarly to other soft fruits, the seasonal availability and short shelf life of fresh blackberries promote their preservation by freezing, drying, canning, or processing into jams, jellies, and juices to prolong storage, ensure availability in various markets and meet consumer demand (Hager et al., 2008; Veberic et al., 2014). Freezing is generally considered to be the least destructive preservation technology for phenolic compounds in berries, and is a recommended pretreatment procedure in berry processing (Wu et al., 2010).

The diverse structures and high content of anthocyanins in the fruit cells of blackberries ensure their black colour intensity (Howard et al., 2012). Anthocyanin stability under freezing conditions and after frozen storage is dependent on the seasonal period of harvest, cultivar and frozen storage period (Veberic et al., 2014). Literature data on the effect of freezing on the chemical properties of the berry are scarce, and they mostly refer to the total contents of some groups of compounds (e.g., phenols and anthocyanins). In view of the above, blackberries should be systematically studied for

nutritional changes in the fruit as induced by freezing and frozen storage.

The objective of this study was to investigate the effect of freezing and frozen storage of blackberries at different ripeness stages on pigment stability, soluble solids and anthocyanin contents, and their relationship.

Materials and methods

Plant material and experimental design

Blackberry (*Rubus* subg. *Rubus* Watson) fruits of three cultivars ('Čačanska Bestrna', 'Loch Ness' and 'Chester Thornless') were harvested at two different stages of ripeness, namely fully ripe and over-ripe. Fully ripe (optimally ripe) fruits were black, glossy and easily picked from the branches. Over-ripe fruits were black, without the characteristic gloss, and fell from the branches at the slightest touch. The investigation was conducted over a two-year period (2013–2014) in an experimental orchard established in 2006 at Gornja Gorevnica (43°53'N, 20°20'E, 290 m alt.) near Čačak, Western Serbia. Blackberries were planted at a spacing of 3.0 m in the row and 1.5 m within the row, and trained to a three-wire trellis. The experiment was laid out in a randomised block design with four replications per cultivar. Fertilisation, weed control and irrigation practices standard for the region were provided during both seasons.

Weather conditions in Čačak were characterised by the mean growing season temperature of 17.0°C and total rainfall of 340.4 mm for the long-term averages (Table 1).

The soil in the blackberry orchard was vertisol, moderately supplied with organic matter (2.92%) and poor in N (0.11%); soil pH in KCl 0.01 mol L^{-1} was 4.98. The contents of available soil P and K were 4.64 and 29.23 mg 100 g^{-1}, respectively. Under the agro-environmental conditions in the Republic of Serbia, blackberries have their best performance in warm pre-mountainous areas with high air humidity levels during the growing season, particularly at the fruit maturation stage (Nikolić and Milivojević, 2015).

After harvest, blackberry fruits were taken to the laboratory and randomly divided into three treatments: 1) harvest treatment, fresh fruits were immediately analysed; 2) fruits were stored in a freezer at -20°C for 7 days; and 3) fruits were stored in a freezer at -20°C for 1 month. Four replications per treatment were performed, each replication containing 100 blackberry fruits. Samples were tested for soluble solids and total anthocyanins in both harvesting seasons. Fruits were collected at the same sampling date. Data were expressed as two-year mean values.

Determination of PD, SSC and TAC

PD was determined by counting fruits showing PD (fruits with red drupelets that cover more than 80% of fruit) in a sample of 400 fruits (4 replications of 100 fruits) after two freezing periods (7 and 30 days). The rate of PD was expressed in %.

SSC was determined by a digital refractometer (Carl Zeiss, Jena, Germany) at 20°C, and data were expressed in %. TA content was measured by the previously described pH-differential method (Torre and Barritt, 1977; Liu et al., 2002). Briefly, 20 g of ground fruits were blended with 40 mL of an extracting solvent (95% ethanol/1.5 N HCl, 85:15). The extract was collected by filtration with an additional 30 mL of the washing solvent. The residue was soaked with 70 mL of the extracting solvent, and the extract was collected after 2 h. The total extracts were pooled and brought up to a volume of 200 mL. A UV/VIS spectrophotometer (PU 8740 UV/VIS, England) and a disposable cell of 1 cm path length were used for spectral measurements at 510 and 700 nm. Pigment content was calculated as milligrams of cyanidin-3-glucoside per 100 g fresh weight (mg cyn-3-glu 100 g^{-1} FW) using an extinction coefficient of 26,900 L cm^{-1} mol^{-1} and molecular weight of 449.2 g mol^{-1}.

Statistical analysis

The data were presented as mean ± standard error of the mean (SE). Differences between means were compared by Duncan's Multiple Range test in three-way and two-way analysis of variance (ANOVA) using the MSTAT-C statistical computer package (Michigan State University, East Lansing, MI, USA). Differences at $p \leq 0.05$ were considered significant.

Table 1. Mean monthly and growing season temperatures and rainfall in Čačak in 2013 and 2014.

Month	Air temperature (°C)			Rainfall (mm)		
	2013	2014	LTA*	2013	2014	LTA*
January	0.5	1.9	0.3	124.9	61.6	46.6
February	-3.2	3.0	2.3	70.9	79.6	42.6
March	6.1	5.6	6.8	25.1	73.0	49.5
April	12.1	13.2	11.5	61.2	41.6	52.3
May	15.3	16.9	16.8	125.4	111.6	75.7
June	22.0	18.4	20.0	41.2	96.8	89.4
July	25.1	21.5	21.5	43.0	58.0	69.1
August	23.9	22.7	21.2	2.8	35.0	44.3
September	19.6	14.1	16.7	15.0	50.0	58.8
October	12.8	12.7	11.4	53.2	1.0	69.4
November	8.3	7.4	6.0	20.0	60.0	49.6
December	-0.2	0.1	1.4	87.6	12.6	59.6
Mean or total of growing season	18.7	17.1	17.0	341.8	394.0	459.0

* Long-term average (48-year average, i.e., 1965–2013 period).

Results

Evaluation of PD

The analysis of variance showed significant effects of all treatments and their interaction (cultivar/ripeness stage/frozen storage period) on the rate of PD in blackberry fruits and the number of fruits showing no PD (Table 2).

The rate of PD and the number of berries showing pigment stability ranged from 0.00 ± 0.00 to 73.69 ± 13.16 and 59.33 ± 0.88 to 92.33 ± 0.88, respectively. Across cultivars, the lowest PD was recorded in 'Loch Ness' (the highest number of fruits with no PD), and the highest in 'Čačanska Bestrna' blackberries (the lowest number of fruits showing pigment stability). Irrespective of cultivar, the rate of PD was higher at the fully ripe stage. On the other hand, the rate of PD of blackberry fruits was lower during 7 days of frozen storage.

The analysis of interaction effects of all treatments (cultivar/ripeness stage/frozen storage period) showed that the rate of PD was highest in fully ripe 'Čačanska Bestrna' fruits during 30 days of frozen storage, and lowest in over-ripe fruits of 'Loch Ness' and 'Chester Thornless' in the same frozen storage period (30 days).

Fruit quality parameters

Cultivar, ripeness stage and frozen storage period significantly affected SSC and TAC (Table 3). The SSC of the fruit ranged from 9.51 ± 0.21 to 10.93 ± 0.23% and TAC from 81.20 ± 2.77 to 150.07 ± 6.88 mg cyn-3-glu 100 g^{-1} FW. Across cultivars, 'Loch Ness' fruits gave the highest SSC and TAC, whereas the lowest SSC and TAC were measured in 'Čačanska Bestrna' fruits. It was also found that SSC and TAC were higher in over-ripe blackberry fruits.

The analysis of the interaction effect of all treatments (Figure 1) revealed that SSC was highest in over-ripe 'Loch Ness' fruits without freezing (0 days), and lowest in fully ripe 'Čačanska Bestrna' fruits during 30 days of frozen storage.

Throughout the experimental period, it was only the stage of ripeness (higher SSC in over-ripe fruits) that had a stimulating effect on SSC, whereas cultivar and frozen storage period produced no significant effect.

TAC is presented in Figure 2, showing an almost identical trend across storage periods. The lowest TAC was recorded in fully ripe 'Čačanska Bestrna' fruits during 30 days of frozen storage, and the highest in over-ripe 'Loch Ness' fruits without freezing (0 days). During different frozen storage pe-

Table 2. Influence of cultivar, ripeness stage and frozen storage period on the rate of pigment degradation in blackberry fruits.

Treatment			Rate of pigment degradation in fruit (%)	Number of fruits with no pigment degradation
Cultivar (A)				
'Loch Ness'			4.90 ± 1.63 c	85.17 ± 1.36 a
'Chester Thornless'			13.26 ± 3.29 b	81.92 ± 4.09 b
'Čačanska Bestrna'			35.24 ± 7.51 a	60.83 ± 7.90 c
Ripeness stage (B)				
Fully ripe			26.18 ± 5.98 a	69.00 ± 6.06 b
Over-ripe			9.42 ± 2.31 b	82.94 ± 2.48 a
Frozen storage period (C)				
7 days			13.93 ± 1.71b	84.39 ± 1.63 a
30 days			21.67 ± 6.69 a	67.56 ± 6.14 b
Cultivar × Ripeness stage × Frozen storage period (A × B × C)				
'Loch Ness'	Fully ripe	7 days	11.00 ± 0.58 de	89.00 ± 0.58 ab
		30 days	0.00 ± 0.00 e	89.00 ± 0.58 ab
	Over-ripe	7 days	8.59 ± 2.91 de	81.33 ± 2.33 bc
		30 days	0.00 ± 0.00 e	81.33 ± 2.33 bc
'Chester Thornless'	Fully ripe	7 days	16.33 ± 0.88 cd	83.67 ± 0.88 ab
		30 days	29.04 ± 1.81 b	59.33 ± 0.88 e
	Over-ripe	7 days	7.67 ± 0.88 de	92.33 ± 0.88 a
		30 days	0.00 ± 0.00 e	92.33 ± 0.88 a
'Čačanska Bestrna'	Fully ripe	7 days	27.00 ± 3.21 bc	73.00 ± 3.21 cd
		30 days	73.69 ± 13.16 a	20.00 ± 10.01 f
	Over-ripe	7 days	13.00 ± 1.53 d	87.00 ± 1.53 ab
		30 days	27.27 ± 1.97 bc	63.33 ± 2.85 de
ANOVA				
A			*	*
B			*	*
C			*	*
A × B			*	*
B × C			*	*
A × C			*	*
A × B × C			*	*

Values within each column followed by the same small letter are not significantly different at $p \leq 0.05$ by LSD test.

riods, it was evident that TAC showed a downward trend in all blackberry cultivars, as well as at different stages of ripeness. In contrast, the treatment without freezing resulted in a higher TAC in blackberry fruits.

Discussion

PD

Freezing prolongs the shelf life of fruits, but thawing causes softening of the fruit, i.e., changes in its consistency (Petzold and Aguilera, 2009). Given that consumers' assessment of fruit quality is based on colour (Giusti and Wrolstad, 2003), which in blackberries is governed by a high content of different anthocyanins in fruit cells (Howard et al., 2012), it is necessary to retain colour uniformity for increased acceptance of the fruit in the market. The market value of 'Čačanska Bestrna' blackberries can considerably decline due to the instability of the black colour of fruits during frozen storage, and due to the tendency of the fruit to release juice, i.e., poor transportability (Nikolić and Milivojević, 2015). The observed rate of pigment degradation in blackberries in the present study supports the report of De Ancos et al. (2000) that genotype, stage of fruit ripeness and abiotic factors can impact the freezing effect. Specifically, the highest rate PD was determined in 'Čačanska Bestrna' blackberries and the lowest in 'Loch Ness' blackberries, and the significance of differences was observed among all genotypes analysed. As regards the rate of PD across ripeness stages, significantly higher values were found for fruits sampled at the fully ripe stage, due to the lower TAC, given the findings of Slatnar et al. (2012b) that the beginning of anthocyanin synthesis is parallel to the onset of ripeness stage, as determined through the activity of some enzymes in the phenilpropanoid cycle. When observed across frozen storage periods, a significantly higher rate of PD was obtained after 30 days of frozen storage, consistently with the results of Veberic et al. (2014) showing that the stability of anthocyanins is also affected by frozen storage period, which is, according to Artes (2002) and Stinzing and Carle (2004), attributed to their instability, i.e., degradation during storage, resulting in decreased colour intensity of fruits and their processed products. Blackberry cultivars showed no patterns of variation in the rate of PD

Table 3. Influence of cultivar, ripeness stage and frozen storage period on soluble solids and total anthocyanins in blackberry fruits with no pigment degradation.

Treatment	Soluble solids content (%)	Total anthocyanin content (mg cyn-3-glu 100 g^{-1} FW)
Cultivar (A)		
'Loch Ness'	10.93 ± 0.23 a	150.07 ± 6.88 a
'Chester Thornless'	10.53 ± 0.20 b	102.55 ± 8.83 b
'Čačanska Bestrna'	9.51 ± 0.21 c	81.20 ± 2.77 c
Ripeness stage (B)		
Fully ripe	9.71 ± 0.17 b	109.45 ± 6.74 b
Over-ripe	10.93 ± 0.32 a	113.09 ± 8.72 a
Frozen storage period (C)		
0 days	10.86 ± 0.24 a	143.88 ± 9.43 a
7 days	10.07 ± 0.29 b	101.40 ± 7.52 b
30 days	10.04 ± 0.22 b	88.54 ± 5.93 c
ANOVA		
A	*	*
B	*	*
C	*	*
A × B	ns	*
B × C	*	*
A × C	ns	*
A × B × C	*	*

Values within each column followed by the same small letter are not significantly different at $p \leq 0.05$ by LSD test; ns: non-significant differences.

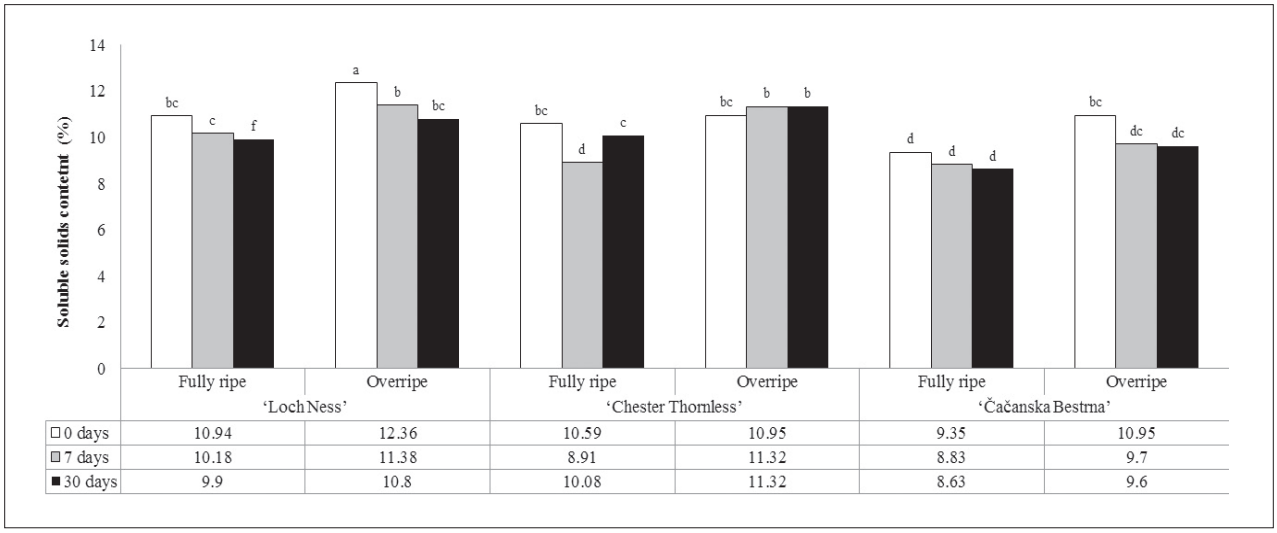


Figure 1. Soluble solids content (A × B × C).
* The same small letters represent non-significant differences at P ≤ 0.05 by LSD test.

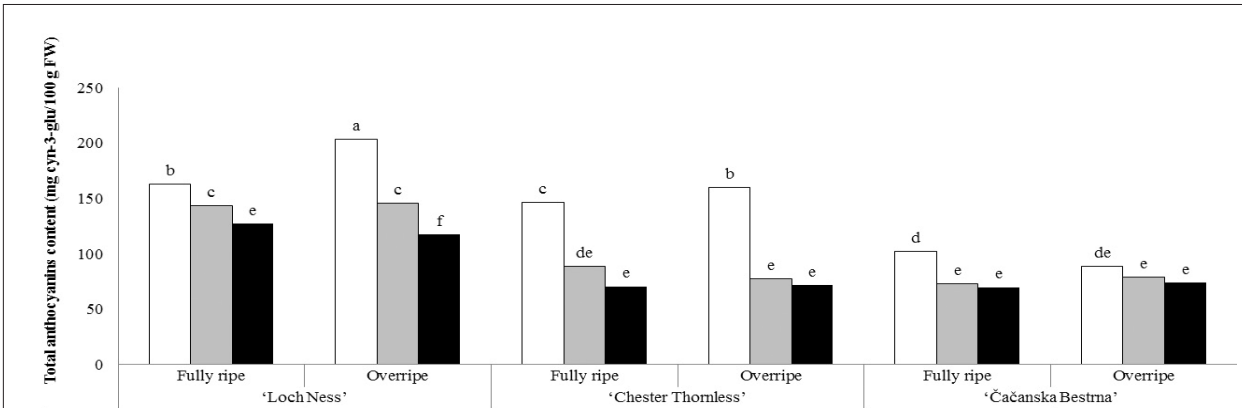


Figure 2. Total anthocyanin content (A × B × C).
* The same small letters represent non-significant differences at $P \leq 0.05$ by LSD test.

in all treatments. When analysing literature data, Veberic et al. (2014) found that the effect of processing and different storage procedures on the chemical properties of primary and secondary metabolites (anthocyanins for the most part) in blackberries was commonly studied in juiced, pureed and canned fruits.

Fruit quality parameters

The soluble solid and total anthocyanin contents of the over-ripe blackberry fruits (without PD and with PD) are showing stability in frozen storage conditions in all tested cultivars. As shown by Kopjar et al. (2012), specific sugars such as glucose can have a positive effect on anthocyanin content in blackberry juice by preventing their degradation during storage. Namely, in berries as well as in other fruits, anthocyanins begin to appear at onset of ripening when a higher activity of certain enzymes of phenylpropanoid pathway is observed and continue to build up during maturation (Slatnar et al., 2012a). The evolution of anthocyanins parallels the accumulation of sugars, but no direct relationship has been established (Cadot et al., 2011). Significantly higher SSC and TAC were obtained in fruits sampled at the over-ripe stage, with 'Loch Ness' fruits having the highest content and 'Čačanska Bestrna' the lowest. Frozen storage period had a significant effect on the contents of the fruit quality parameters tested, which continuously decreased during freezing, due to cianidin-3-glucoside degradation in the chemical reactions during freezing (Kopjar et al., 2012). The content and structure of anthocyanins in small fruits are determined by genotype and different environmental factors (Kähkönen et al., 2003). In the present study, the SSC and TAC in blackberry fruits which showed pigment stability were largely governed by the interaction cultivar × ripeness stage × frozen storage period and, therefore, the effect of individual factors cannot be independently analysed. Katalinić (2006) found that anthocyanin degradation in the fruit is due to increased content of sugar intermediates (furfural and hydroxymethylfurfural - HMF) formed as enzymatic and non-enzymatic browning products, i.e., as the result of an unfavourable effect of temperature during processing and storage. The same author further stressed the protective role of disaccharides in anthocyanin stability before they break down into monosaccharides, with sugars ordered as follows according to their effect: glucuronic acid, fructose, sucrose, lactose, maltose, glucose and gluconic acid. As shown by Kopjar et al. (2012), individual sugars, especially glucose, in blackberry fruits prevent anthocyanin degradation during storage (freezing). In their study on the effect of long-term frozen storage on the chemical properties of different kinds of berries, Poiana et al. (2010) found that total anthocyanins in blueberries and raspberries decreased by 3% and 9% after 6 and 10 months of storage, respectively. When analysing changes in the contents of anthocyanins and other chemical compounds in blackberry fruits due to different freezing methods, Veberic et al. (2014) determined lower values of TAC in 'Čačanska Bestrna' and 'Loch Ness' than in the present study, whereas the values for 'Chester Thornless' were similar.

Conclusion

The present study indicates that frozen storage period (0, 7, and 30 days) is one of the easiest methods for storing blackberries for off-season use and an appropriate way of preserving plant metabolites in fruits. Drastic chemical changes occur in the fruits during their ripening and the contribution of soluble solids content to stability of total anthocyanins was generally dependent on their levels in blackberries. The results of this study show that the ripeness stage significant increases soluble solids content and total anthocyanins content has been noted in over-ripe berries compared to fully ripe fruit resulting in lower rates of pigment degradation. Therefore, our results can be useful for further studies on food processing and human metabolomic research.

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Addresses of authors:
Žaklina Karaklajić-Stajić[1,*], Mihailo Nikolić[2], Jelena Tomić[1], Rade Miletić[1] and Marijana Pešaković[1]
[1] Fruit Research Institute Čačak, Kralja Petra I, 9, 32000 Čačak, Serbia
[2] Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Belgrade, Serbia
* Corresponding author