

COMPOSITION AND CONCENTRATION OF PHENOLIC COMPOUNDS OF 'AUKSIS' APPLE GROWN ON VARIOUS ROOTSTOCKS

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The trial was carried out at the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry in 2013–2015. Cv. 'Auksis' was tested on 12 rootstocks: B.396, B.9, M.9, M.26, P 22, P 59, P 61, P 62, P 66, P 67, PB.4, and Pure 1. Accumulation of phenolic compounds depended on fruit yield and average fruit weight. On average, significantly lower concentration among rootstocks occurred when apple trees had abundant yield and fruits were smaller. On average chlorogenic acid constituted 50% and total procyanidins 28% of total phenols in 'Auksis' fruits. Flavonoid concentration most depended on rootstock and the highest variation was recorded. More than 50% difference occurred between the highest total flavonoid concentration in apples on PB.4 and the lowest on M.9 rootstocks. Low variability of total procyanidin concentration among rootstocks was observed. Differences between the highest and lowest concentration was 15%. Total concentration of phenolic compounds differed among rootstocks by 29–35% depending on the year. Differences in accumulation of phenolic compounds depended on rootstock genotype but not on yield or fruit weight. PB.4 and P 67 rootstocks had the highest, and M.9, P 62 and M.26 had the lowest concentration of total phenol in 'Auksis' fruits.

Key words: bioactive substances, fruit, *Malus x domestica* Borkh., yield.

INTRODUCTION

Apples along with bananas are the most important fruits in the world. Apples also are distinguished by the highest amounts of phenolic compounds among fruits (Sun *et al.*, 2002). Different apple cultivars as a source of variation of phytochemical content have been widely tested (Wolf *et al.*, 2003; Ceymann *et al.*, 2012; Liaudanskas *et al.*, 2015; Wang *et al.*, 2015; Xu *et al.*, 2016).

Accumulation of phenolic compounds in apple fruits depends on climatic and technological factors: weather conditions (Scalzo *et al.*, 2005), sun exposure (Awad *et al.*, 2001), fruit position on the tree (Hagen *et al.*, 2007; Feng *et al.*, 2014) and fertilisation practice (Awad and de Jager, 2002). In spite of numerous studies of apple phenolic compounds, effect of rootstock on the accumulation of bioactive substances in apple fruits has not been widely tested. Rootstock choice is one of the most important decision in modern apple growing. Rootstock has obvious influence on apple tree development, precocity, and fruit quality, and it

regulates transport of minerals in the tree. Studies on rootstocks of other orchard plants revealed great differences between rootstocks in accumulation of phenols, for example in lemon (Gil-Izquierdo *et al.*, 2004) and cherry (Bauer *et al.*, 1989), but studies of apple rootstock regarding phenolic compound concentration in fruits have been conducted only in Estonia and Lithuania (Mainla *et al.*, 2011; Kviklys *et al.*, 2014).

The aim of the study was to evaluate apple rootstock effect on accumulation of phenolic compounds in 'Auksis' fruits and to test impact of the rootstock on the variability and stability of bioactive substance concentration.

MATERIALS AND METHODS

The trial was conducted in a productive orchard at the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry in 2013–2015. Trees were planted at spacing 4 × 1.5 m in 2005 and trained as slender spindle.

Pest and disease management was carried out in conformity with the rules of integrated plant protection. Rootstocks for this trial were chosen based on their popularity worldwide and suitability for intensive apple growing: super-dwarf rootstocks P 22, P 59, P 61, and PB.4 (Belarusian selection), dwarf rootstocks M.9, P 62, P 66, P 67, B.396, B.9, and Pure 1 (Latvian selection), and semi-dwarf rootstock M.26 (Kviklys *et al.*, 2013).

Rootstocks were grafted with cv. 'Aukšis' and planted in a randomised block design, with four replicates and three trees per plot. Twenty randomly selected fruits from each replication were taken for biochemical analysis and carefully mixed. Twenty-five randomly selected fruits were chosen from the bulk sample and analysed in three replicates of composite samples.

Preparation of samples. Each apple was cut into slices of equal size (up to 1 cm in thickness), and the stalks and the seeds were removed. Slices were immediately frozen in a freezer (−35 °C) with air circulation and then lyophilised with a ZIRBUS sublimator 3×4×5/20 (ZIRBUS technology, Bad Grund, Germany) at a pressure of 0.01 mbar (condenser temperature, −85 °C). The lyophilised apple slices were ground to a fine powder using a Retsch 200 mill (Haan, Germany). Loss on drying before analysis was determined by drying the apple lyophilisate in a laboratory drying oven to complete evaporation of water and volatile compounds (temperature, 105 °C; difference in weight between measurements up to 0.01 g) and by calculating the difference in raw material weight before and after drying. Data were recalculated for absolute dry lyophilisate weight.

Chemicals. All the solvents, reagents, and standards used were of analytical grade. Acetonitrile and acetic acid were obtained from Sigma-Aldrich GmbH (Buchs, Switzerland) and ethanol from Stumbras AB (Kaunas, Lithuania). Hyperoside, rutin, quercitrin, phloridzin, procyanidin B1, procyanidin B2, and chlorogenic acid standards were purchased from Extrasynthese (Genay, France), (+)-catechin and (−)-epicatechin from Fluka (Buchs, Switzerland), and avicularin and isoquercitrin from Chromadex (Santa Ana, USA). Deionised water, produced by the Crystal E HPLC (Adrona SIA, Riga, Latvia) water purification system, was used.

Extraction. An amount of 2.5 g of lyophilised apple powder (exact weight) was weighed, added to 30 mL of ethanol (70%, v/v), and extracted in a Sonorex Digital 10 P ultrasonic bath (Bandelin Electronic GmbH & Co. KG, Berlin, Germany) for 20 minutes at 40 °C. The conditions of extraction (type of extraction, duration, temperature, solvent and its concentration) were chosen considering the results of extraction optimisation. The obtained extract was filtered through a paper filter; the apple lyophilisate on the filter was washed twice with 10 mL of ethanol (70%, v/v) in a 50-mL flask. The extract was filtered through a membrane filter with a pore size of 0.22 µm (Carl Roth GmbH, Karlsruhe, Germany).

Instrumentation and chromatographic conditions. Hyperoside, isoquercitrin, rutin, avicularin, quercitrin, (+)-catechin, (−)-epicatechin, procyanidin B1, procyanidin B2, phloridzin, and chlorogenic acid standards were dissolved in ethanol (96.3%, v/v). A mixture of these standards, diluted with ethanol (96.3%, v/v) to 1/2, 1/4, 1/8, and 1/16 of the initial concentration, was prepared. The initial mixture of standards and the diluted solutions were injected into the HPLC system. A calibration curve with five concentration levels was constructed for each compound. The analyses of standard solutions were performed in triplicate. The concentrations of phenolic compounds identified in the apple extracts were within the limits of calibration curves.

A Waters 2695 chromatograph equipped with a Waters 2998 photodiode array (PDA) detector (Waters, Milford, USA) was used for HPLC analysis. Chromatographic separation was managed, chromatograms were recorded, and data were processed with the Empower[®] v.2.0. software (Waters, Milford, USA). Chromatographic separations were carried out by using a YMC-Pack ODS-A (5 µm, C₁₈, 250 × 4.6 mm i.d.) column equipped with a YMC-Triart (3 µm, C₁₈, 10 × 3.0 mm i.d.) precolumn (YMC Europe GmbH, Dinslaken, Germany). The column was operated at a constant temperature of 25 °C. The volume of the extract being investigated was 10 µL. The flow rate was 1 mL/min, and gradient elution was used. The mobile phase consisted of 2% (v/v) acetic acid in water (solvent A) and 100% (v/v) acetonitrile (solvent B). The following conditions of elution were applied: 0–30 minutes, 3–15% B; 30–45 minutes, 15–25% B; 45–50 minutes, 25–50% B; and 50–55 minutes, 50–95% B. Detection was simultaneously performed at three wavelengths: 280 nm (dihydrochalcones, catechins, procyanidins), 320 nm (phenolic acids), and 360 nm (flavonols). The spectrum of UV-visible light was recorded from 200 to 600 nm. All the phenolic compounds were identified by comparing their retention times and spectra with those of the standard compounds. The concentration of phenolic compounds was calculated based on the peak areas by using standard compounds (Fig. 1).

The data on the main traits were subjected to the analysis of variance. Significance of differences between treatment (rootstock) means was evaluated using the LSD test at $p < 0.05$.

RESULTS

The total phenol concentration in 'Aukšis' apples varied between rootstocks and years (Fig. 2). Differences between rootstocks were not the same during years of the study. In 2013, the lowest concentration of total phenolic compounds was recorded on P 62 rootstock (3741 µg/g DW) and the highest — on P 59 rootstock (5745 µg/g DW). In 2014, the lowest concentration was on M.9 (3147 µg/g DW) and the highest on Pure 1 (4690 µg/g DW). In 2015, the lowest concentration was recorded again on M.9 (3891 µg/g DW) and the highest on P 62 (5475 µg/g DW).

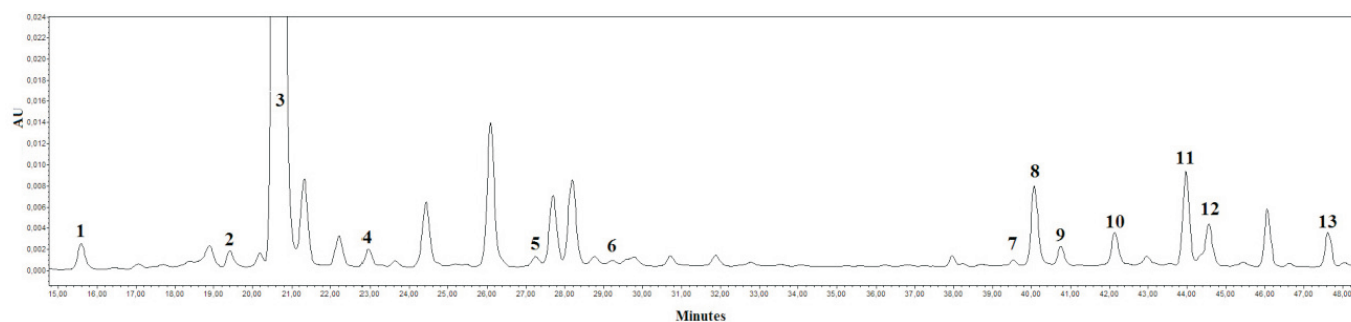


Fig. 1. Chromatogram of the ethanol extract of cv. 'Auksis' on M.9 rootstock fruit sample ($\lambda = 320$ nm). Numbers indicate the peaks of analytes: (1) procyanidin B1; (2) (+)-catechin; (3) chlorogenic acid; (4) procyanidin B2; (5) (-)-epicatechin; (6) procyanidin C1; (7) rutin; (8) hyperoside; (9) isoquercitrin; (10) reinotrin; (11) avicularin; (12) quercitrin; (13) phloridzin.

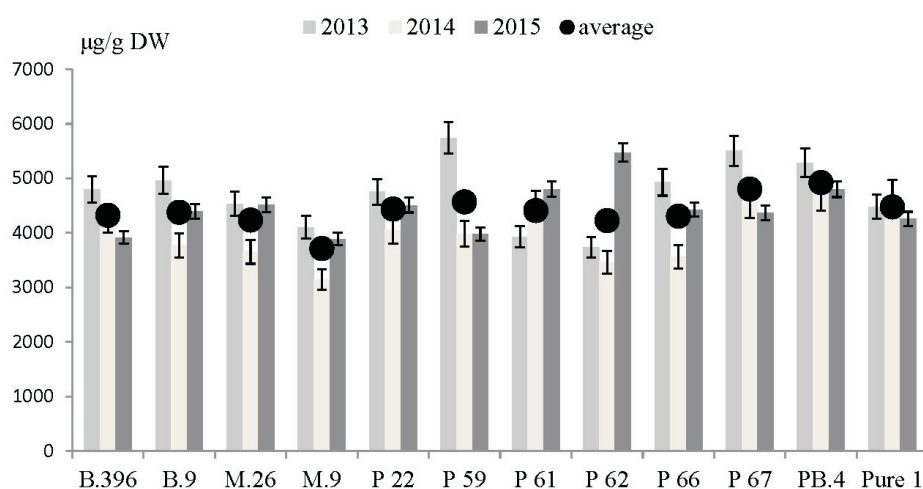


Fig. 2. Rootstock effect on the total phenol concentration in 'Auksis' apple fruits, $\mu\text{g/g DW}$. Error bars indicate least significant differences at the 5% probability level for each year separately.

On average over three years, PB.4 and P 67 rootstocks accumulated the highest (4924 and 4804 $\mu\text{g/g DW}$ respectively), while M.9 rootstock accumulated the lowest concentration of total phenols (3714 $\mu\text{g/g DW}$).

Significant differences between rootstocks were observed in concentration of different phenolic compounds (Table 1). The highest concentration of quercetin glycosides (hypero-

side, isoquercitrin, rutin, avicularin, and quercitrin) was found in fruits from trees grown on super dwarf rootstocks PB.4, P 59, and Pure 1. In contrast, the most vigorous trees on M.26 rootstock accumulated the lowest concentration of quercetin glycosides. M.9 rootstock significantly differed from other rootstocks in low accumulation of epicatechin, procyanidins, phloridzin, and chlorogenic acid. 'Auksis' ap-

Table 1

ROOTSTOCK EFFECT ON THE AVERAGE CONCENTRATION OF PHENOLIC COMPOUNDS IN 'AUKSIS' APPLE FRUITS, $\mu\text{g/g DW}$, AVERAGE OF 2013–2015.

Rootstock	Hyperoside	Isoquercitrin	Rutin	Avicularin	Quercitrin	Catechin	Epicatechin	ProcyanidinB1	ProcyanidinB2	Phloridzin	Chlorogenic acid
B.396	87.7	16.5	14.6	74.6	57.9	165	373	209	1099	123	2103
B.9	108.6	20.3	15.6	89.8	68.1	158	359	200	1006	112	2241
M.26	47.7	11.5	11.8	50.1	44.1	174	377	228	1031	149	2112
M.9	59.9	14.1	12.6	56.0	45.5	149	326	140	924	98	1890
P 22	77.9	16.6	15.2	70.4	56.1	174	399	223	1021	107	2276
P 59	121.8	25.6	22.0	105.9	78.4	164	359	218	1040	116	2320
P 61	91.8	18.4	15.3	79.8	64.5	153	403	197	1085	115	2188
P 62	74.6	16.0	13.4	70.8	57.5	167	391	179	1013	120	2123
P 66	79.2	16.8	13.7	72.6	59.2	127	379	230	1026	133	2171
P 67	81.6	17.5	14.5	74.4	58.6	182	434	235	1162	146	2398
PB.4	129.2	25.9	19.0	103.2	78.5	140	429	239	1082	116	2562
Pure 1	127.2	25.6	17.9	102.1	79.0	168	391	170	1052	153	2191
Mean	90.6	18.7	15.5	79.1	62.3	160	385	206	1045	124	2215
LSD ₀₅	14.6	5.1	4.3	12.2	9.9	14.9	39.3	18.6	81.5	10.7	140.6

Table 2

VARIATION OF PHENOLIC COMPOUND CONCENTRATION IN 'AUKSIS' APPLE FRUITS DURING THREE GROWING SEASONS ON DIFFERENT ROOTSTOCKS, AVERAGE OF 2013–2015, VARIATION COEFFICIENT V%.

Rootstock	Hyperoside	Isoquercitrin	Rutin	Avicularin	Quercitrin	Catechin	Epicatechin	ProcyanidinB1	ProcyanidinB2	Phloridzin	Chlorogenic acid	Total phenol content
B.396	36	35	14	27	16	4	3	41	31	14	5	13
B.9	13	6	26	17	17	11	9	38	26	23	15	14
M.26	43	31	35	33	29	4	15	53	28	65	12	12
M.9	53	48	25	38	25	2	14	22	34	26	12	14
P 22	29	20	16	32	19	15	8	51	23	27	6	8
P 59	34	34	42	21	12	16	16	65	38	10	21	22
P 61	45	22	43	37	27	13	20	39	10	37	16	10
P 62	30	13	27	25	22	26	32	40	38	35	24	26
P 66	47	29	19	39	19	43	6	57	30	34	13	16
P 67	56	41	27	47	30	17	3	46	19	31	13	13
PB.4	12	21	2	22	28	40	5	44	13	12	13	6
Pure 1	20	14	38	18	20	23	5	9	13	37	10	5
Mean	35	26	26	30	22	18	11	42	25	29	13	13

ples grown on P 67 and PB.4 rootstocks had the richest concentration of the latter phenolic compounds.

During the trial period the most stable concentration (coefficient of variation (V%) = 5 and 6) of total phenolic compounds was found in trees on PB.4 and Pure 1 rootstocks (Table 2). The most variable concentration of total phenolic compounds in three years of investigations was recorded in apples on P 62 and P 59 rootstocks (V% = 26 and 22, respectively). On average for the rootstocks, procyanidin B2 and hyperoside concentrations were the most variable, while epicatechin and chlorogenic acid concentrations were the most stable (V% = 11 and 13, respectively). Variation of separate phenolic compound concentration depended on rootstock. Very high variation (V% > 50) of hyperoside was recorded in 'Auksis' apples grown on M.26 and P 67 rootstocks, procyanidin B1 — on P 59 and phloridzin — on M.26 rootstock. The lowest variation (V% 10) of isoquercitrin concentration during three years was recorded on B.9, rutin on PB.4, catechin on B.396, B.9, P 22, and M.26, epicatechin on B.396, B.9, P 22, P 66, P 67, PB.4, and Pure 1, procyanidin B1 on Pure 1, and chlorogenic acid on B.396 and P 22 rootstocks.

The average apple yield was obtained in 2014 and fruits this year were distinguished by the lowest concentration of total phenols (Table 3). When the average yield was low, significantly higher concentration of phenols accumulated. Such relationships were not found for average fruit weight.

On average for the rootstocks, tree growth was negatively correlated ($r = -0.55$) with phenol concentration in fruits (Fig. 3).

On average for three years, fruit colour was positively correlated with the total phenol concentration ($r = 0.62$) (Fig. 4), although fruits from P 67 rootstock had only intermediate blush. However, by separate years, strong correlation

Table 3

AVERAGE ANNUAL APPLE YIELD, FRUIT WEIGHT AND TOTAL CONCENTRATION OF PHENOLIC COMPOUNDS IN 'AUKSIS' APPLES, AVERAGE OF 12 ROOTSTOCKS

	2013	2014	2015	LSD ₀₅
Total phenols, µg/g DW	4731	4025	4445	298
Yield, kg/tree	5.4	29.6	9.8	4.6
Fruit weight, g	157	138	183	24

between fruit colour and the total concentration of phenolic compounds was established only in 2013 ($r = 0.69$), medium in 2014 ($r = 0.42$) and no correlation in 2015 ($r = 0.09$) (data not presented).

DISCUSSION

Cv. 'Auksis' is one of the most popular apple cultivars grown in commercial and amateur orchards in Baltic countries (Univer *et al.*, 2010; Lepsis *et al.*, 2014). Along with good yield and excellent fruit quality, cv. 'Auksis' accumulates the highest concentration of phenolic compounds in comparison with some other commercially important apples cultivars grown in Lithuania (Liaudanskas *et al.*, 2015). Our study demonstrated that total phenol concentration even could be increased by selecting the proper rootstock genotype, thereby enhancing the value of 'Auksis' fruits.

Cv. 'Auksis', like many apple cultivars, has biennial fruiting habit (Kviklys *et al.*, 2016). Average apple yield during the investigation period differed significantly and it was inherent for all rootstocks tested. Average total phenol concentration was significantly lower in high yielding years and higher when apple had less abundant yield. Despite this overall phenomena, annual yield differences did not affect accumulation of phenols in fruits grown on B.396, P 61,

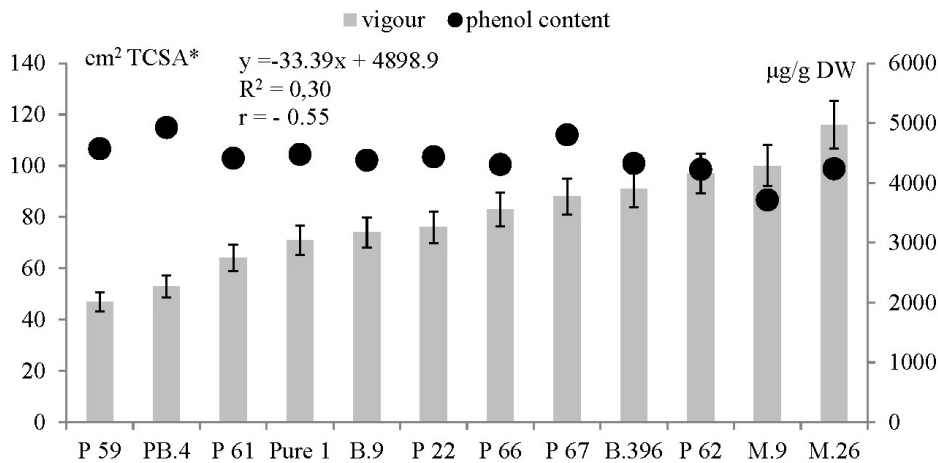


Fig. 3. Rootstock effect on tree vigour and total phenol concentration in apple fruits, average 2013–2015. Rootstocks are arranged in order of increasing tree vigour increasing order. *TCSA, trunk cross sectional area. Error bars indicate least significant differences between rootstocks in TCSA at the 5% probability level.

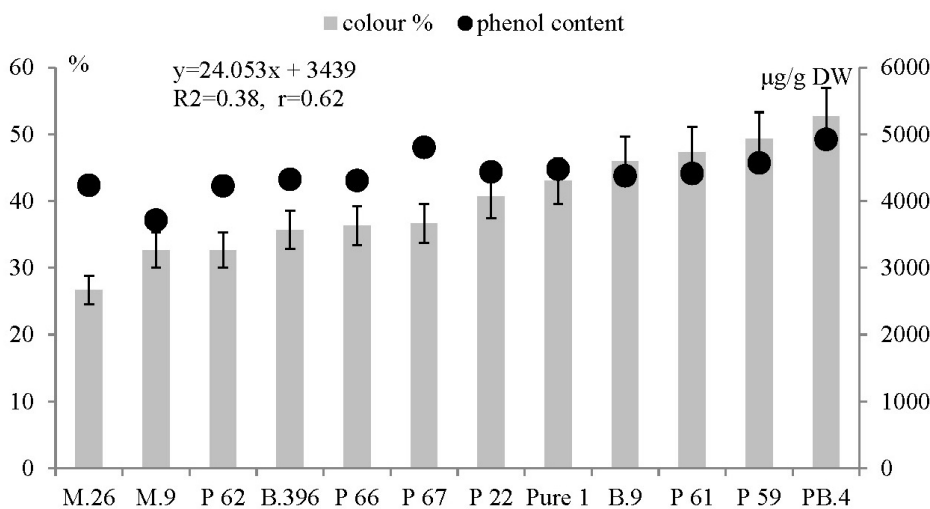


Fig. 4. Rootstock effect on fruit blush and total phenol concentration, average 2013–2015. Rootstocks are arranged in order of increasing fruit blush, average 2013–2015

Error bars indicate least significant differences between rootstocks in fruit blush at the 5% probability level.

P 67, and Pure 1 rootstocks, indicating that the accumulation of phenols is more related to rootstock genotype.

Accumulation of phenolic compounds depends on climatic conditions. Differences of their concentration in apple fruits could be caused also by other factors (as yield, nutrition, soil management (Awad and de Jager, 2002; Wang and Lin, 2003; Mittelstras *et al.*, 2006; Lanauskas *et al.*, 2016). Depending on the year, the total concentration of phenolic compounds differed among rootstocks by 29–35%. Choosing the right rootstock with stable phenol concentration could be achieved in the future. In our trials, rootstocks Pure 1 and PB.4 demonstrated very stable accumulation of total phenolic compounds despite variable yield and vegetation conditions. In order to achieve stability of individual phenolic compound concentration, certain rootstock cultivar combinations should be considered, as results from the trial with cultivar ‘Ligol’ demonstrated slightly different rootstock behaviour (Kviklys *et al.*, 2014).

Flavonoids play an important role in tree growth processes and auxin transport (Buer *et al.*, 2010). It has been established that more colourful fruits contain more quercetin glycosides. Lu *et al.* (1999) demonstrated significantly higher concentrations of quercetin glycosides in fully coloured fruits from the top of the tree and low concentrations in not coloured fruits from the inner canopy. Similar results

were obtained by Hagen *et al.* (2007) and Feng *et al.* (2014). Our trial results confirm these findings. ‘Auksis’ apples from trees on M.26 rootstock were at least colourful and the content of quercetin glycosides in apples on this rootstock was the lowest. In contrast, significantly higher concentration of the main flavonoids were found in apples grown on PB.4 and P 59 rootstocks, which also positively influenced fruit colouration. More intensive fruit colour had effect on quercetin glycoside concentration but not on total phenolic compound concentration.

Mainla *et al.* (2011) reported that vigorous rootstocks accumulated less phenolic compounds in a trial performed in Estonia. However, there were only three rootstocks tested. Medium negative correlation established between tree growth and concentration of total phenolic compounds in our trial, but rootstock genotype played more important role in accumulation of phenolic compounds than induced tree vigour. The most vigorous rootstock M.26 had an intermediate concentration of phenolic compounds, while super dwarf PB.4 and dwarf P 67 had the highest concentration. The lowest accumulation of total phenols was detected in fruits grown on dwarf M.9 rootstock, the vigour of which did not differ significantly from P 67, which had the highest phenol concentration in ‘Auksis’ apples. Negative influence of M.9 rootstock on total phenol concentration was recorded also in a previous trial (Kviklys *et al.*, 2014).

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ŠĶIRNES 'AUKSIS' ĀBOLU SASTĀVS UN FENOLU SAVIENOJUMU SATURS, AUDZĒJOT UZ DAŽĀDIEM POTCELMIEM

Pētījums veikts Lietuvas lauksaimniecības un mežsaimniecības pētījumu centra Dārzkopības institūtā 2013.–2015. gadā. Šķirne 'Auksis' tika pārbaudīta uz 12 potcelmiem: B.396, B.9, M.9, M.26, P 22, P 59, P 61, P 62, P 66, P 67, PB.4 un Pure 1. Fenolu savienojumu uzkrāšanās bija atkarīga no ražas lieluma un vidējā augļu svara. Vidēji uz visiem potcelmiem ievērojami zemāks fenolu saturs novērots tad, kad ābeles bagātīgi ražoja un augļi bija sikāki. Vidēji šķirnes 'Auksis' augļos hlorogēnskābe veidoja 50% no kopējie procianidīni 28% no kopējā fenolu satura. Flavonoīdi bija visvairāk atkarīgi no potcelma, un tiem novērotas lielākās variācijas. Novērota vairāk nekā 50% atšķirība starp augstāko kopējo flavonoīdu saturu augļos uz potcelma PB.4 un zemāko saturu uz M.9. Kopējiem procianidīniem atšķirības atkarībā no potcelma bija mazas. Starpība starp augstāko un zemāko saturu bija 15%. Kopējais fenolu savienojumu saturs atšķirās starp potcelmiem par 29–35% atkarībā no gada. Atšķirības fenolu savienojumu uzkrāšanās daudzumā ietekmēja potcelma genotips, bet ne ražas lielums vai augļu svars. Potcelmi PB.4 un P 67 deva augstāko, bet M.9, P 62 un M.26 zemāko kopējo fenolu saturu šķirnes 'Auksis' augļos.