The study of polyphenolic spectrum of Prunus spinosa and their spontaneous hybrids in relation to fruit polyphenols accumulation in conditions of Slovakia

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Abstract

Nowadays, more and more attention has been focused on underutilized fruit species, including blackthorn (*Prunus spinosa* L.) fruit. In the territory of Slovakia, spontaneous hybrids such as *P. ×dominii*, *P. ×fruticans*, and *P. ×fechtnerii* have also occurred, studied only in respect of ploidy and genome size. The aim of the presented paper was to examine and compare the fruit variability in anatomy, localization of bioactive substances, and the content of polyphenols and antioxidant activity of *P. spinosa* fruit, including its spontaneous hybrids. The results of the research proved that variability in fruit anatomy and differences in the polyphenolic spectrum, TPC content, and antioxidant activity among hybrids can be evaluated as a result of spontaneous hybridization with cultivated species of *Prunus.*

1. Introduction

The genus *Prunus* includes 400–430 species belonging to the *Rosaceae* family (Ullah et al. 2020). The species within this genus are spread all over the world, although only 98 species can be commercially utilized (Komakech and Kang 2009). Besides cultivated species, many lesser-known, underutilized, or wild plant species can be a significant source of nutritionally valuable substances, such as polyphenolic compounds (Juríková et al. 2019, 2021; Ivanišová et al. 2025). One of them is *Prunus spinosa* L. (blackthorn plum), which represents a valuable source of phenolic antioxidants (flavonoids, anthocyanins, phenolic acids) valued for their antioxidant, anti-inflammatory, antiproliferative, antiviral, and antibacterial properties (Negrean et al. 2023).

Widely, *P. spinosa* agg. creates a dominant component in xerothermic shrub vegetation communities of *Berberidion vulgaris* Br.-Bl. 1950 alliance (class *Rhamno-Prunetea* Rivas Goday et Borja Carbonell ex Tüxen 1962) (Jarolímek et al. 2008) in biocorridors on the edges of agricultural land in southwestern Slovakia (Muráňová et al. 2012b).

P. spinosa agg. is a very variable species. The variability of the taxon is manifested in several morphological characters, including fruits (Muráňová et al. 2012a; Žgančíková et al. 2012; Vander Mijnsbrugge et al. 2016; Gavrilă et al. 2017). This manifestation of traits largely arises as a result of hybridization with the cultivated species *P. insititia* and *P. domestica*. Cytometric analyses revealed that *P. spinosa* agg. consists of both the native species *P. spinosa* as well as the tetraploid hybrids *P. ×dominii, P. ×fruticans, P. ×schurii*, and the pentaploid taxon *P. ×fechtnerii* (Žabka et al., 2018). Although tetraploid taxa are generally characterized by fruit set, variable fruit production may occur due to the presence of introgressed forms in hybrid populations of *P. spinosa* agg. (Muráňová et al. 2011; Žabka et al. 2015). Compared to tetraploid taxa, the pentaploid taxon *P. ×fechtnerii* is characterized by a rare fruit set (Muráňová et al. 2012a).

In addition, from the perspective of the use and processing of plant products, knowledge of the structure of plant parts as well as the localization of metabolites in the tissues of individual organs is of considerable importance. The structure of the pericarp is mostly studied in more detail for cultivated taxa of the genus *Prunus* (Konarska 2015a, b; Vio-Michaelis et al. 2020; Mohd et al. 2022;); however, there is only limited knowledge about the structure of the fruit for *Prunus spinosa* (Boz et al. 2018).

Prunus species represent valuable sources of bioactive compounds (over 500) (Liu et al. 2020; Baires et al. 2012), with the predominance of polyphenolic compounds (Jang et al. 2016; Acero et al. 2018). Although several studies of nutrient content have been conducted in *P. spinosa* agg. (Berk et al. 2020) or artificial hybrids of *P. spinosa* with *P. cerasifera* and *P. domestica*, which have shown changes in nutrient content (Minev and Balev 2002), there have been no more detailed analyses within the aggregate taxon *P. spinosa* or its natural hybrids. There is an assumption that the hybridization of blackthorn with cultivated species also resulted in changes in the content of metabolites, and hybrids will be characterized by different levels of these substances. In terms of polyphenol content as the prevailing

compounds in fruit, there has been no comparison of the variability of fruit content in spontaneous hybrids of *P. spinosa* in autochthonous blackthorn populations.

2. Materials and methods

2.1 Plant Material Collection

The ripe fruits of the taxa *P. spinosa*, *P. × dominii*, *P. × fruticans*, *P. × schurii*, and *P. × fechtnerii* were collected at the Jelenec locality (48°23'47.2"N 18°12'34.2"E, Nitra district) on October 26, 2021. Fruits for microscopic analyses were processed immediately after collection, and fruits for chemical analyses were stored in a freezer at -20°C until use. Stands of investigated Prunus taxa are located in Nitrianska pahorkatina Uplands, a geomorphological sub-unit of the Podunajská pahorkatina Uplands. They form a strip of land between the Váh and Nitra rivers. The Nitra Uplands are a geomorphological sub-unit of the Podunajská pahorkatina Uplands. They form a strip of land between the Váh and Nitra rivers. The area is intensively used for agriculture; the original oak stands have been converted into arable land, meadows, and pastures on luvisol soils (Gajdoš 1990). From the climatic point of view, the locality is classified as a moderately warm area characterized by an average of less than 50 summer days per year (with a daily maximum air temperature ≥ 25°C), and the average July air temperature ≥ 16°C (Lapin 2002). The average temperature in January ranges from 1 to 4°C, in July 18 to 20°C. The average precipitation is 600 to 700 mm per year (Šiška and Repa 2002).

2.2 Microscopic Analysis

The fleshy parts (skin and mesocarp) of the pericarp were used for microscopic observations. The cut pericarp slices, 3–5 mm thick, were fixed in formaldehyde-ethanol-acetic acid fixative. This was followed by embedding the material in paraffin through an ethanol-butanol series. The material was cut using a CUT 4055 MICROTEC rotary microtome. Sections with a thickness of 7–10 µm were prepared and stained with safranin and fast green (Němec 1962). Five fruits from each taxon were processed for the evaluation of morphological characters. The following fruit traits were evaluated: thickness of the epidermis, cuticle thickness, thickness of the wax layer, hypodermis thickness, exocarp thickness, and mesocarp thickness. The hypodermis and sub-hypodermic layers morphologically distinct from the mesocarp were considered exocarp. The epidermis with cuticle and waxes was evaluated separately. The fruits of *P. × fechtnerii* were not used for anatomical observation due to lack of material. Microscopic images were taken using an Olympus DP23 camera. Measurement of morphometric features was performed in the Cell Sens Standard (Olympus Corporation, Japan).

2.3 Extraction of Sample

Immediately after culling, the fruits were frozen and stored at -40°C. The extraction was performed according to Hakimuddin et al. (2008) with modifications as provided below. The frozen fruits were homogenized in 90% methanol (2 ml/g) and subsequently extracted at 4°C for 30 minutes. After the extraction, centrifugation at 1990 rpm for 10 minutes was used to separate the supernatant, and the sediment was subjected to a new extraction. This process was repeated three times. The supernatants containing phenolic compounds were dried using a Laborota 4011 digital rotary evaporator (Heidolph, Germany) and stored at -20°C. The content of total polyphenols an individual polyphenolic compounds were detected in fresch weith (FW) in five replications and calculated on dry weith (DW).

2.4 Total Polyphenol Content (TPC)

A standard solution of tannin (Sigma Aldrich) was prepared by dissolving 50 mg of tannin in 100 ml of water. Using a pipette, the solution was placed in six flasks (50 ml) in volumes of 0.2, 0.3, 0.4, and 0.5 ml. The extract (1 ml) was

added to seven flasks and dissolved as needed. Distilled water (20 ml) and Folin-Ciocalteu reagent (1 ml) were added to each flask. After three minutes, a 20% Na2CO3 solution (5 ml) was added. The solutions were mixed, and distilled water was added to a final volume of 50 ml. After 30 minutes, the color intensity compared to the control (no tannin) was measured at 700 nm.

2.5 HPLC Analyses of Individual Polyphenolic Compounds

The amount of polyphenolic compounds – phenolic acids and flavonoids – was detected by the HPLC method. For the analysis of individual polyphenols, we used the method described by de Quiros et al. (2010) with some modifications: polyphenols were analyzed by HPLC Dionex 3000 (USA) with UV-VIS detection and Chromeleon 7 system software. Chromatographic separation was carried out on a Phenomenex Kinetex C18 column (150 × 4.6 mm). The flow rate was 1 ml/min. The injection volume was 10 µl. The separation was performed at room temperature (30°C). The detector was set at 275 nm.

2.6 Antioxidant Activity (AA) of Fruits

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed according to the previously described methods (Brand-Williams et al. 1995) with some modifications. Stock solutions were prepared by dissolving 24 mg of DPPH in 100 ml of methanol and then stored at -20°C until needed. Working solutions were prepared by mixing 10 ml of the stock solution with 45 ml of methanol to obtain an absorbance of 1.1 ± 0.02 units at 515 nm using a Libra S6 spectrophotometer. Fruit extracts (150 µl) were allowed to react with the DPPH solution (2,850 µl) for 1 hour in the dark. Following this, absorbance was measured again at 515 nm. Antioxidant activity was calculated as a decrease in the absorbance value using the formula: (%) = (A0 – A1/A0) × 100%, where A0 is the absorbance of the blank (without the sample), and A1 is the absorbance of the mixture containing the sample. The absorbance results were converted using a standard calibration curve and expressed as ascorbic acid equivalents (AAE) (Rupasinghe et al. 2006). This protocol was repeated three times for each fruit extract.

A solution of 3.5 mmol Abts and 0,06 mol.l^{-1} $K_2S_2O_8$ were prepared by dissolving ABTS in deionized water. ABTS radical was generated by mixing the solutions in a ratio of 50:1 and left in the dark for 16 hours, then mixing with acetate buffer pH 4.3 in a ratio of 39:1. 4 ml of the reaction mixture was stirred with 50 µl of sample, left in the dark for 30 minutes and measured at 734 nm. Buffer was used as a blank, trolox as a standard. The decrease in absorbance was expressed as a percentage (Re et al. 1999).

2.7 Statistical Analyses

Morphological data were analyzed by analysis of variance (ANOVA) using Tukey's HSD test ($P \leq 0.01$) (Statistica software, version 10, StatSoft, Inc., Tulsa, OK, USA).

3. Results

3.1 Chemical analyses

The results of the study showed that *Prunus spinosa* had the highest content of total polyphenols (4.33 ± 0.02 GA mg/g FW), mirroring the antioxidant activity (AA) determined by the DPPH and ABTS methods (5.71 ± 0.58 mg TROLOX/g and 7.57 ± 0.17mg TROLOX/g, respectively). On the other hand, the lowest total polyphenol content (TPC) was found in *P. ×fechtneri* (1.96 ± 0.13 GA mg/g FW), and the lowest AA was observed in *P. ×fruticans* (1.92 ± 0.10 mg TROLOX/g by the DPPH method) and *P. ×fechtneri* (2.92 ± 0.08 mg TROLOX/g by the ABTS method).

Evaluation of the polyphenolic spectrum of P. spinosa and its spontaneous hybrids

Polyphenolic spectrum of *P. spinosa* and its spontaneous hybdrids were evaluated phenolic acid and flavonoids.

Phenolic acids

The leading and most structurally diversified group of polyphenols were represented by phenolic acids. Among phenolic acids, the highest accumulation was detected in neochlorogenic acid in samples of *P. ×fechtneri* (2196.96 µg/g FW; 11,108.71 µg/g DW). The lowest content of this acid was found in samples of *P. spinosa* (1015.61 µg/g FW; 3306.18 µg/g DW). The second most abundant was chlorogenic acid, with values ranging from 13.05 µg/g FW; 65.98 µg/g DW (*P. ×fechtneri*) to 63.04 µg/g FW; 258.46 µg/g DW (*P. ×fruticans*).

In terms of content, the third in order was protocatechuic acid, achieving the maximum concentration in *Prunus spinosa* (37.08 µg/g FW; 120.71 µg/g DW), comparable with *P. ×dominii* samples (36.72 µg/g FW; 153.40 µg/g DW). p-hydroxybenzoic acid was present in the fruit of *P. ×fruticans* at a concentration of 10.41 µg/g FW; 39.53 µg/g DW, similar to *P. spinosa* (10.03 µg/g FW; 32.65 µg/g DW). The lowest was detected in *P. ×fechtneri* (4.76 µg/g FW; 24.09 µg/g DW). Ellagic acid was accumulated in the highest amount in *P. ×fruticans* (18.65 µg/g FW; 70.85 µg/g DW), while in *P. ×dominii* it was detected only at a concentration of 0.9 µg/g FW; 3.77 µg/g DW. Caffeic acid was mostly present in *P. fruticans* (17.53 µg/g FW; 66.60 µg/g DW), with the lowest values found in *P. ×dominii* (9.83 µg/g FW; 41.04 µg/g DW).

Ferulic acid was the predominant acid in samples of *P. ×fechtneri* (18.34 µg/g FW; 92.72 µg/g DW), with the lowest values examined in *P. ×dominii* (4.14 µg/g FW; 17.30 µg/g DW). The highest accumulation of sinapic acid was assayed in samples of *P. ×fruticans* (8.77 µg/g FW; 32.55 µg/g DW), similar to *P. ×dominii* (8.56 µg/g FW; 35.76 µg/g DW). Syringic acid prevailed in samples of *P. spinosa* (7.37 µg/g FW; 24 µg/g DW), while in *P. ×fechtneri* it was observed only at a concentration of 1.37 µg/g FW; 6.97 µg/g DW. The highest accumulation of gallic acid was examined in *P. spinosa* samples (7.26 µg/g FW; 23.63 µg/g DW), with the lowest in *P. ×fechtneri* (0.53 µg/g FW; 2.63 µg/g DW).

Among phenolic acids, the lowest values were found for *p*-trans-coumaric acid, which prevailed in samples of *P. ×fechtneri* (3.35 µg/g FW; 16.92 µg/g DW), and *o*-coumaric acid, which was present in trace amounts in *P. ×dominii* (0.29 µg/g FW; 1.23 µg/g DW), similar to *P. spinosa* (0.28 µg/g FW; 0.91 µg/g DW). In our studies, vanillic acid was not detected.

Flavonoids and resveratrol

Catechin, epicatechin, and rutin have been reported to have protective effects against *diabetes*, while other flavonoids, including myricetin, quercetin, and kaempferol, exhibit antihypertensive activity (Negrean et al. 2023). The content of quercetin was similar in all evaluated samples, ranging from 8.88 µg/g FW; 33.74 µg/g DW in samples of *Prunus ×fruticans* to 9.92 µg/g FW; 58.18 µg/g DW in samples of *P. ×fechtneri*. Similarly, the highest accumulation of rutin was observed in *Prunus ×fruticans* (5.34 µg/g FW; 20.28 µg/g DW), with the lowest in *P. ×dominii* (1.39 µg/g FW; 5.82 µg/g DW). The highest concentration of resveratrol was typical for Prunus *spinosa*, with similar content assayed in samples of *P. ×fechtneri* and *P. ×fruticans* (0.14 µg/g FW; 0.69 µg/g DW; 0.17 µg/g FW; 0.63 µg/g DW).

Catechin was detected only in *Prunus ×dominii* (1.79 µg/g FW; 7.5 µg/g DW) and in *P. ×fruticans* (3.44 µg/g FW; 13.07 µg/g DW). Conversely, epicatechin was present in all samples, with the highest concentration in *P. ×dominii* (22.44 µg/g FW; 93.73 µg/g DW) and the lowest in samples of *P. ×fruticans* (4.68 µg/g FW; 17.78 µg/g DW).

3.2 Microscopic Analyses

Microscopic observations revealed that the fruit surface of the studied taxa is covered by an epidermis with a continuous layer consisting of cuticle and waxes (Fig. 1). However, the taxa differ in the shape of the epidermal cells. While the epidermal cells of *P. spinosa* have longer tangential walls, in *P. ×dominii* and *P. ×fruticans*, the radial walls of the epidermal cells reach the largest dimensions. As a result, the shape of the epidermal cells of *P. spinosa* is mostly elongated, while the epidermal cells of *P. ×dominii* are more broadly ovoid to round (Fig. 1, 3). During fruit ripening, we observed an accumulation of anthocyanins mostly in the skin cells in the form of granular structures of various sizes, which in mature fruits are concentrated in the central vacuole of the cells (Fig. 2, 3). In mature blackthorn fruits, the vacuole fills almost the entire volume of the epidermal cells and is characterized by intense coloration. The most intensely colored vacuoles with the highest anthocyanin content in ripe fruits are observed in *P. spinosa*. The gradual accumulation of pigments and the lowest concentration of anthocyanins are observed in *P. ×dominii*. This observation is also consistent with the results of biochemical analyses. The skin of blackthorn fruits is covered with a continuous layer of cuticle. In addition, we observed that the cuticular matrix penetrates between the radial walls of the cuticular cells. The thinnest cuticle of the studied taxa is found in *P. ×dominii*, with a thickness of 2.95 µm, while the cuticle thickness of the other two taxa exceeds 3 µm (Table 3).

Table 1
The content of TPC and AA of *Prunus spinosa* L. and its spontaneous hybrids

Taxa / parameters	Content TPC (GA)	DPPH (TROLOX)	ABTS (TROLOX)
FW	[mg.g^{-1}] FW	[mg.g^{-1}] FW	[mg.g^{-1}] FW
P. spinosa	4.33 ± 0.02	5.71 ± 0.58	7.57 ± 0.17
P. ×dominii	2.69 ± 0.09	2.37 ± 0.06	4.82 ± 0.14
P. ×fruticans	2.21 ± 0.08	1.92 ± 0.10	3.88 ± 0.26
P. ×fechtnerii	1.96 ± 0.13	2.02 ± 0.10	2.92 ± 0.08

Table 2
The content of polyphenolic compounds of *Prunus spinosa* L. and its spontaneous hybrids

Taxa	*P. spinosa*		*P. ×dominii*		*P. ×fruticans*		*P. ×fechtnerii*	
dry matter (%)		30.72		19.78		26.33		19.78
Polyphenolic compounds	FW	DW	FW	DW	FW	DW	FW	DW
gallic acid	7.26 ± 0.1	23.63	3.20 ± 0.1	13.38	3.69 ± 0.1	14.03	0.53 ± 0.0	2.69
3,4-dihydroxybenzoic (protocatechuic) acid	37.08 ± 0.2	120.71	36.72 ± 0.2	153.40	21.13 ± 0.0	80.27	17.17 ± 0.2	86.80
neochlorogenic acid	1015.61 ± 0.3	3306.18	1887.97 ± 0.1	7886.45	1561.17 ± 4.7	5930.35	2196.96 ± 5.4	11108.71
4-hydroxybenzoic	10.03 ± 0.0	32.65	9.01 ± 0.1	37.65	10.41 ± 0.3	39.53	4.76 ± 0.1	24.09
epigallocatechin	833.04 ± 7.9	2711.85	620.94 ± 9.9	2593.81	1371.41 ± 3.0	5209.50	1136.88 ± 7.9	5748.51
catechin	N.D.		1.79 ± 0.1	7.50	3.44 ± 0.3	13.07	N.D.	
vanillic acid	N.D.		N.D.		N.D.		N.D.	
chlorogenic acid	33.66 ± 0.1	109.59	23.03 ± 0.2	96.21	68.04 ± 0.3	258.46	13.05 ± 0.1	65.98
caffeic acid	15.49 ± 0.0	50.44	9.83 ± 0.1	41.04	17.53 ± 0.3	66.60	13.65 ± 0.3	69.04
syringic acid	7.37 ± 0.2	24.00	5.68 ± 0.0	23.73	5.32 ± 0.0	20.20	1.37 ± 0.0	6.95
epicatechin	18.93 ± 0.0	61.64	22.44 ± 0.3	93.73	4.68 ± 0.1	17.78	5.21 ± 0.4	26.37
trans-p-coumaric acid	2.70 ± 0.0	8.78	2.91 ± 0.0	12.15	3.83 ± 0.0	14.54	3.35 ± 0.0	16.92
ferrulic acid	8.94 ± 0.1	29.09	4.14 ± 0.1	17.30	17.40 ± 0.2	66.11	18.34 ± 0.4	92.72
sinapic acid	8.33 ± 0.0	27.13	8.56 ± 0.0	35.76	8.57 ± 0.0	32.56	6.24 ± 0.0	31.57
elagic acid	1.46 ± 0.0	4.75	0.90 ± 0.1	3.77	18.65 ± 0.0	70.85	6.46 ± 0.1	32.66
rutin	3.75 ± 0.0	12.22	1.39 ± 0.0	5.82	5.34 ± 0.1	20.28	4.48 ± 0.1	22.65
t-2-hydroxycinnamic acid (o-coumaric ac.)	0.28 ± 0.0	0.91	0.29 ± 0.0	1.23	0.23 ± 0.0	0.88	N.D.	
protocatechuic	15.49 ±	50.42	12.39 ±	51.76	4.09 ±	15.53	3.26 ±	16.46

Taxa	*P. spinosa*		*P. ×dominii*		*P. ×fruticans*		*P. ×fechtnerii*	
acid etylester	0.6		0.0		0.1		0.0	
resveratrol	0.81 ± 0.0	2.62	0.56 ± 0.0	2.32	0.17 ± 0.0	0.63	0.14 ± 0.0	0.69
trans-cinnamic acid	N.D.		N.D.		N.D.		N.D.	
kaempferol	N.D.		N.D.		N.D.		N.D.	
quercetin	9.04 ± 0.6	29.41	9.11 ± 0.0	38.06	8.88 ± 0.2	33.74	9.92 ± 0.1	50.18

Table 3
Fruit morphometry of *Prunus spinosa* L. and its spontaneous hybrids

Taxon	Thicknees of the individual layers of the fruits (µm)					
	Wax	**Cuticle**	**Epidermis**	**Hypodermis**	**Exocarp**	**Flesh**
Prunus spinosa	5.58 ± 1,32^{b}	3,33 ± 0,52^{b}	15,24 ± 1,99^{a}	19,13 ± 2,9^{a}	36,67 ± 5,65^{b}	2319,43 ± 328,04^{b}
Prunus ×dominii	6,96 ± 1,17^{a}	2,95 ± 0,40^{a}	18,52 ± 1,98^{b}	27,25 ± 3,8^{c}	75,68 ± 9,58^{a}	2886,87 ± 127,98^{a}
Prunus ×fruticans	7,16 ± 1,14^{a}	3,11 ± 0,43^{b}	15,83 ± 1,92^{a}	23,26 ± 2,73^{b}	79,49 ± 8,88^{a}	2781,98 ± 141,84^{a}

The fruits of blackthorn are characterized by the deposition of waxes on the skin surface. The epicuticular wax layer of ripe fruits consists of 2–4 layers formed from scales and granules. The thinnest wax layer, with a thickness of 5.58 µm, is found in the taxon *P. spinosa*. The hybrid taxa *P. ×dominii* and *P. ×fruticans* are characterized by a thicker wax layer reaching 6.96 and 7.16 µm, respectively (Table 3).

Under the epidermis, the hypodermis is developed, consisting of two layers of cells. The hypodermis cells are slightly flattened, with elongated tangential walls, resulting in a mostly oval cell shape. The hypodermis cells are characterized by thickened cell walls, giving the layer the character of collenchymatic tissue. We found that the assayed taxa differ from each other in the thickness of the hypodermis. The species *P. spinosa* has the thinnest hypodermis, while the hybrids can be characterized by a thicker hypodermis (Table 3). Similar to the epidermis, we also observed the presence of granular structures corresponding to anthocyanin bodies in the cells of this layer. However, these structures occur in much smaller quantities compared to epidermal cells (Fig. 4).

Under the hypodermis, 4 to 6 layers of parenchymatous cells are developed, similarly flattened, often more pointed than the hypodermic cells. However, these cells are larger in size compared to the hypodermis. Unlike the hypodermis, these cells do not contain the granular material corresponding to the anthocyanin bodies observed in the epidermis and hypodermis, but groups of plastids are present in the cells (Fig. 5).

Below the exocarp, we can observe the thickest layer of blackthorn fruit, the parenchymatous mesocarp, extending to the sclerenchymatous endocarp. We noticed that the mesocarp is formed by two distinct layers of parenchymatous cells. The outer part of the mesocarp consists of 4 to 6 layers of large parenchymatous cells of a round shape with increased volume towards the interior of the fruit.

In the case of the thickness of the hypodermis together with the sublayer hypodermis, defined as the exocarp, we found that the studied species differ from each other in the thickness of this layer. We observed the thinnest exocarp in the fruits of *P. spinosa*, while in the hybrids the thickness of this part is almost twice as thick (Table 3). In addition, calcium oxalate druses are present in the exocarp cells of all taxa.

Under the exocarp, we can see the thickest layer of the pericarp, the parenchymatous mesocarp, extending up to the sclerenchymatous endocarp. We observed that the mesocarp of the fruit is formed by two distinct layers of parenchymatous cells. The outer part of the mesocarp consists of 4 to 6 layers of large parenchymatous cells of a round shape, the volume of which increases towards the interior of the fruit. We observed the accumulation of a large amount of granular material in the vacuoles of this layer. The inner part of the mesocarp, about 6–8 layers thick, consists of cells with noticeably elongated tangential walls. The cells of this layer contain dark-colored vacuoles containing polyphenolic substances (Fig. 6).

Comparing the thickness of the fleshy part of the pericarp between taxa, we found that *P. spinosa* has clearly the smallest flesh thickness compared to its hybrids (Table 3).

4. Discussion

The structure of the pericarp of *Prunus* fruits corresponds to that of a drupe (Cerri and Reale 2020), characterized by a fleshy outer pericarp and a sclerenchymatic endocarp. Similarly, fruit development and the accumulation of metabolites in individual fruit layers show common features for several species of the genus *Prunus*, especially the localization of pigments, which is concentrated in the fruit skin (Vignati et al. 2022).

The resistance of fruits to external factors and the length of fruit storage depend on the structure of the surface tissues. The thickness of the epidermis, the presence of cuticle, and waxes have a decisive influence on these properties (Yeats and Rose 2013). Waxes also contribute to the overall appearance of the fruit, and their higher presence, causing a paler shade of dark fruits, may increase their attractiveness more than the content of pigments in the skin (Yan et al. 2023).

The observed differences in the shape and size of epidermal cells and the cuticle thickness of hybrid blackthorns correlate with data on the variability of these traits found in *P. spinosa* from several locations in Romania (Boz et al. 2018). Similar to our results regarding the intraspecific variability of the evaluated traits, the mentioned authors attribute these differences to the influence of genotype, fruit maturity at the time of harvest, and regional climatic and soil conditions.

The skin cells of *P. spinosa* hybrids are characterized by the presence of a central vacuole containing anthocyanin compounds. Anthocyanins, as pigments typical of several types of red or blue-colored fruits, have been observed in the skins of many species. Anthocyanins in the epidermis and hypodermis of ripe plum fruits were noted by Konarska (2015a) and Vio-Michaelis et al. (2020). Similarly, in red apples, several morphological types of anthocyanin compounds were observed in the surface layers of the pericarp in the form of intermediate, hemispherical, and spherical types (Ro-Na et al. 2006).

In addition to the epidermis, anthocyanins can be present in the subepidermal cells of fleshy fruits. For example, the highest accumulation of anthocyanins was recorded in the central vacuole of the cells of the subepidermal layer of berries of *Vitis* species (Moskowitz and Hrazdina 1981). However, a high accumulation of anthocyanin compounds in the skin cells and a smaller amount in the subepidermal layer of fruits of the lesser-known species *Lonicera*, *Oxycoccus*, and *Empetrum* was observed by Juríková et al. (2021). The highest polyphenols present in the skin of

Viburnum spp. were also observed by Konarska and Domaciuk (2018). The highest concentration of polyphenols contributing to the highest antioxidant activity of *P. spinosa* corresponds with the highest accumulation of anthocyanins in epidermal and subepidermal layers. This observation confirmed the results of Blando et al. (2004) and Konarska (2015a) that high levels of anthocyanins in *Prunus* possess strong antioxidant properties.

Prunus spinosa and the examined three spontaneous hybrids had mostly accumulated phenolic compounds in the form of large brown-purple deposits mainly in the epidermal and hypodermal cells, while in the parenchymal cells they were present in smaller quantities as granules or fibers. Similar localization of phenolic contents in plum skin at all stages of fruit ripeness was described by Łata et al. (2009) and Treutter et al. (2012). Although most polyphenolic compounds are accumulated in the skin of ripe dark fruits, polyphenols are also present in deeper layers of the fruit, namely in the mesocarp. It has been found that the accumulation of polyphenolic substances occurs already during the earlier developmental stages of flowers and fruits. In the ovary walls of *P. spinosa*, but also in *P. domestica* as well as species of the genera *Cerasus* and *Amygdalus*, their presence was observed not only in epidermal cells but also in the inner layers of the pistil and scattered in the cells of the middle part of the ovary (Chudíková et al. 2012). A similar distribution of polyphenolic substances in the epidermal and hypodermal layers as well as in the parenchymatous cells of the ovary walls in *P. domestica* is reported by Konarska (2015b).

The accumulation of polyphenols in the walls of the ovary and the developing pericarp is typical not only in the fruit anatomy of *Rosaceae* but also in other fruit species, e.g., *Empetrum* sp. (Juríková et al. 2019) or *Oxycoccus* and *Vaccinium* (Baranec et al. 1996). We found that the mesocarp cells of *P. spinosa* fruits are characterized by shape variability, which is also confirmed by Boz et al. (2018). We recorded the maximum accumulation of polyphenolic compounds in the mesocarp of fruits of hybrid taxa of *P. spinosa* mainly in the elongated cells of the innermost layers of the mesocarp. Smaller amounts of these metabolites were also present in the round cells of the outer part of the mesocarp.

According to Guimarães et al. (2013), Jaiswal et al. (2013), Mikulic-Petskovsek et al. (2016), and Popovic et al. (2020), the fruit of the genus *Prunus* contains four main polyphenolic classes, including phenolic acids, anthocyanins, flavonols, and flavanols (condensed proanthocyanidins). The prevalent and most structurally diversified group of polyphenols were phenolic acids - protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, *p*-coumaric, and isomeric chlorogenic acids (Magiera et al. 2022), which is in accordance with the results of our research.

Significant correlations were found between individual phenolic compounds and the antioxidant capacity of *Prunus* sp. examined by the DPPH, ABTS, and FRAP assays, allowing us to conclude that phenolic compounds are the main contributors to the high antioxidant capacity of *Prunus lusitanica* fruits (Abraão et al., 2022). Similarly, *P. spinosa* with the highest content of TPC displayed the highest values of antioxidant activity determined by the DPPH and ABTS methods. The lowest value of TPC in *P. ×fetchneri* corresponded with the lowest values of antioxidant activity examined by the ABTS method.

The similar content of TPC was detected in fresh fruits of *Prunus spinosa* in studies by Magiera et al. (2022) (4.46 mg/g FW). Lower values, ranging from 2.29 to 3.37 mg/g FW, were determined in studies by Mikulic-Petkovsek et al. (2016), Ruiz-Rodríguez et al. (2014), and Sabatini et al. (2020). On the other hand, according to Bayram (2024), the blackthorn extracts obtained via the UAE method exhibited varying values for TPC, ranging from 7.02 to 11.97 mg GAE/g FW. Miodragović et al. (2019) reported values of 2.30–7.59 mg GAE/g, and Popović et al. (2020) reported values of 11.10–30.43 mg GAE/g. Studies by González-de-Peredo et al. (2020) and Ozzengin et al. (2023) resulted in TPC values of 5.56 ± 0.18 mg/g and 4.41 ± 2.47 mg/g, respectively. According to Popović et al. (2020), differences in TPC content and polyphenolic profiles of *Prunus spinosa* can be caused by different geographic areas due to

climatic conditions (Meland et al. 2024). Differences were observed between fruits collected in colder parts of Europe and in the south of the continent. Moreover, the TPC content is extract-dependent in the following way: methanol > methanol-water > water (3.69 mg/g > 2.44 mg/g > 1.01 mg/g) (Varga 2017). Significant differences in TPC content in genotypes of *P. spinosa* subsp. *dasyphylla* were confirmed by Berk et al. (2020).

According to the polyphenolic compounds profiling in blackthorn, the most abundant classes of polyphenols present in blackthorn are hydroxycinnamic acids. Eight representatives of this group were identified by studies of Popović et al. (2020) as protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, *p*-coumaric, and isomeric chlorogenic acids. According to Dedić et al. (2021), caffeic acid, gallic acid, chlorogenic acid, syringic acid, vanillic acid, ferulic acid, and *p*-coumaric acid were identified in blackthorn fruit, which is in accordance with the results of our detection. HPLC analysis of *P. spinosa* fruit shows high chlorogenic and neochlorogenic acid levels (Varga 2017). Similarly, Popović et al. (2020) identified neochlorogenic acid (3-caffeoyl-quinic acid; 3-CQA), 3-p-coumaroyl-quinic acid (3-pCoQA), and chlorogenic acid (5-vaffeoylquinic acid, 5-CQA) as the major polyphenolic acids. Neochlorogenic and chlorogenic acids also prevailed in samples of *P. spinosa*, *P. ×fruticans, P. ×fetchneri*, and *P. ×dominii* fruits.

HPLC analysis by Celik et al. (2017) showed 12.985 mg/kg FW of chlorogenic acid in *Prunus spinosa* fruit. Our hybrids displayed higher content of this acid, with a maximum content of 63.04 µg/g FW in *P. ×fetchneri. P. ×dominii* reached a value of caffeic acid of 9.83 µg/g FW and 41.04 µg/g DW, which is similar to 10.753 mg/kg FW in *P. spinosa*.

Ferulic acid content in *P. ×fetchneri* was 18.34 µg/g FW and 92.72 µg/g DW, while the lowest values were determined in *P. ×dominii* at 4.14 µg/g FW and 17.30 µg/g DW. Celik et al. (2017) found only a concentration of 0.972 mg/kg FW. Similarly, the content of gallic acid and *p*-trans-coumaric acid can be considered higher in all analyzed samples compared to *P. spinosa* samples analyzed by Celik et al. (2017), which were 0.376 mg/kg FW and 2.363 mg/kg FW. On the other hand, our studies did not confirm the presence of vanillic acid, but Celik et al. (2017) confirmed it at a concentration of 0.032 mg/kg FW.

Epicatechin dominated in samples of *P. ×dominii* at 22.44 µg/g FW and 93.73 µg/g DW, representing a higher concentration compared to studies by Celik et al. (2017) at 2.12 mg/kg FW. The main flavonoids in *Prunus spinosa* fruits are quercetin with its glycosides, such as rutin, which can be found in higher concentrations in the peel (Luna-Vanquer et al. 2017). Quercetin and its derivatives (glucoside, galactoside, and rutinoside) found in our samples were also reported as blackthorn constituents by other authors (Guimarães et al. 2013; Veličković et al. 2014; Meschini et al. 2017). Among flavonoids, rutin and quercetin were detected, while kaempferol was not present in all three assayed samples. In our study, quercetin was the most abundant flavonoid, followed by rutin, which represented higher concentrations in all assayed samples compared to the study by Celik et al. (2017), which examined 0.467 mg/kg FW of rutin.

5. Conclusions

Nowadays, it has become increasingly important to find new sources of valuable bioactive substances. In Slovakia, *Prunus spinosa* L. represents a forgotten and underutilized fruit species, and its spontaneous hybrids have not been examined yet. The presented study offers, for the first time, an overview of the anatomical structure of hybrids, involving chemical analyses focused on TPC content, AA of fruit, together with the detection of the polyphenolic spectrum. Anatomical and chemical analyses revealed that hybrids of *P. spinosa* also represent a valuable source of polyphenols, especially phenolic acids, catechin, and quercetin. Variability in morphological traits and differences in

the polyphenolic spectrum and TPC content among hybrids can be concluded as a result of spontaneous hybridization with the cultivated species of *Prunus.*
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Declarations

Anknowledgment

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Authors' Contributions

All authors contributed to the study conception and design. ĽĎ and PE collected data in the field. JM performed chemical analysis. The first draft of the manuscript was written (including the creation of images and tables) by TJ and ĽĎ, and JM and PE commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

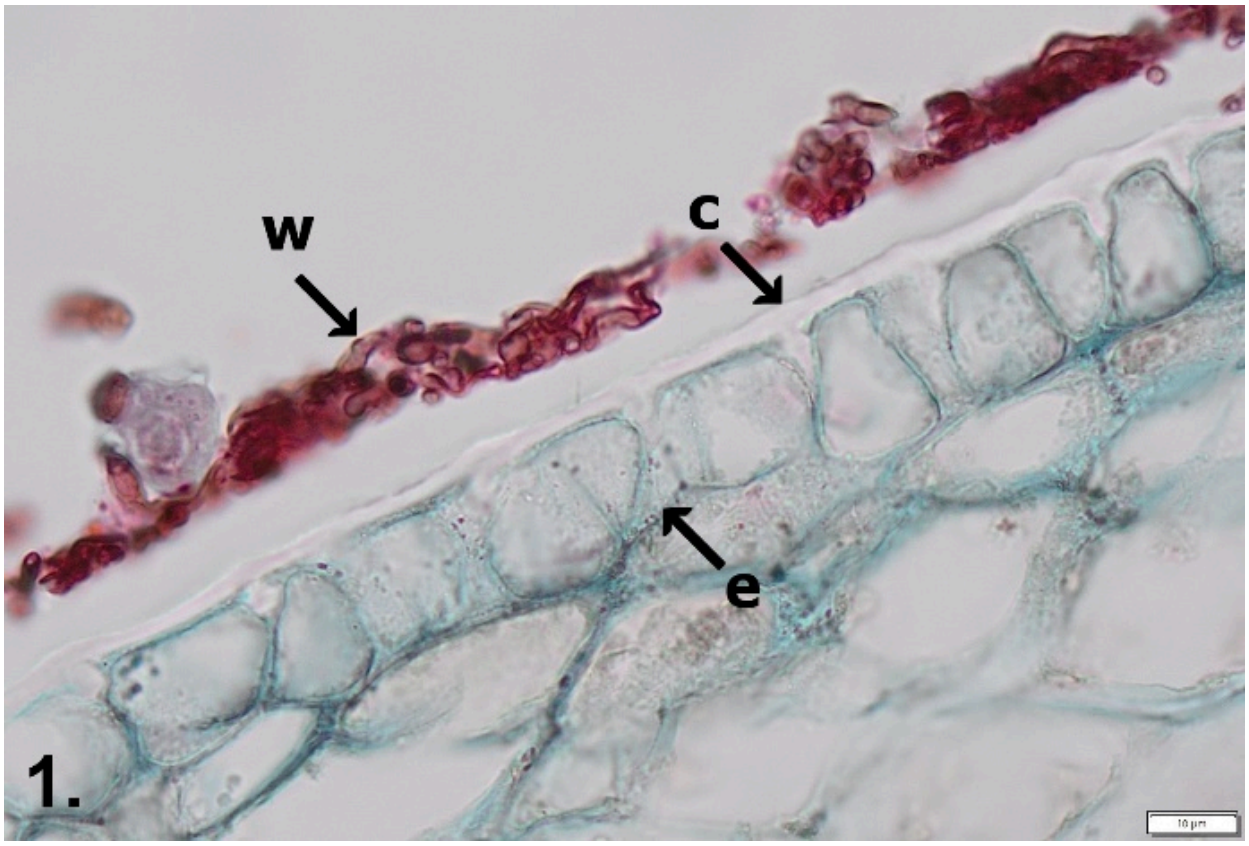

Figure 1

Cross-sections of *Prunus spinosa*fruits and its spontaneous hybrids. 1. *P.* ×*futicans* bar=10 µm, 2. *P. spinosa* bar=10 µm, 3. *P. spinosa* bar=20 µm, 4. *P.* ×*fruticans* bar= 100 µm, 5. P. ×*dominii*, bar=100 µm , 6. *P.* ×*dominii* bar=100 µm; ag – anthocyanis granules, c – cuticle, e - epidermis, h – hypodermis, w – wax

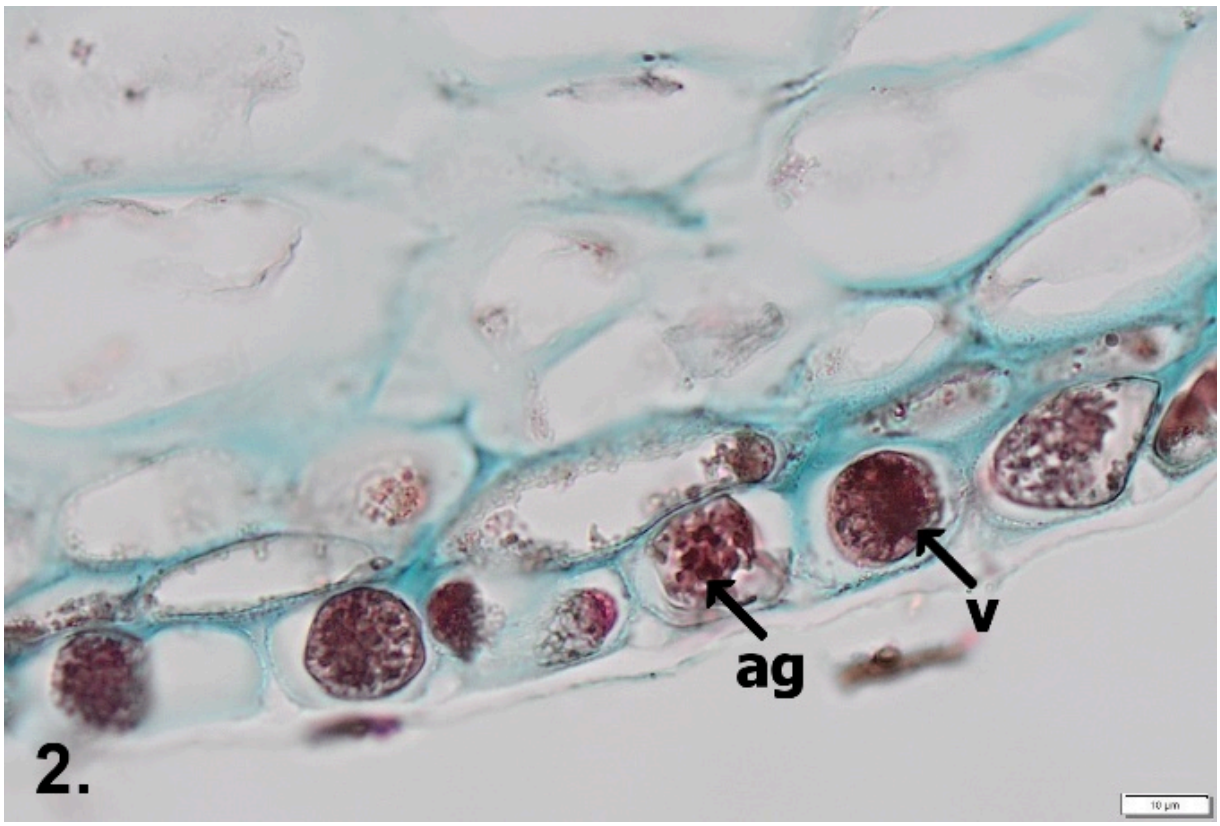

Figure 2

Cross-sections of *Prunus spinosa*fruits and its spontaneous hybrids. 1. *P.* ×*futicans* bar=10 μm, 2. *P. spinosa* bar=10 μm, 3. *P. spinosa* bar=20 μm, 4. *P.* ×*fruticans* bar= 100 μm, 5. P. ×*dominii*, bar=100 μm , 6. *P.* ×*dominii* bar=100 μm; ag – anthocyanis granules, c – cuticle, e - epidermis, h – hypodermis, w – wax

Figure 3

Cross-sections of *Prunus spinosa*fruits and its spontaneous hybrids. 1. *P.* ×*futicans* bar=10 μm, 2. *P. spinosa* bar=10 μm, 3. *P. spinosa* bar=20 μm, 4. *P.* ×*fruticans* bar= 100 μm, 5. P. ×*dominii*, bar=100 μm , 6. *P.* ×*dominii* bar=100 μm; ag – anthocyanis granules, c – cuticle, e - epidermis, h – hypodermis, w – wax

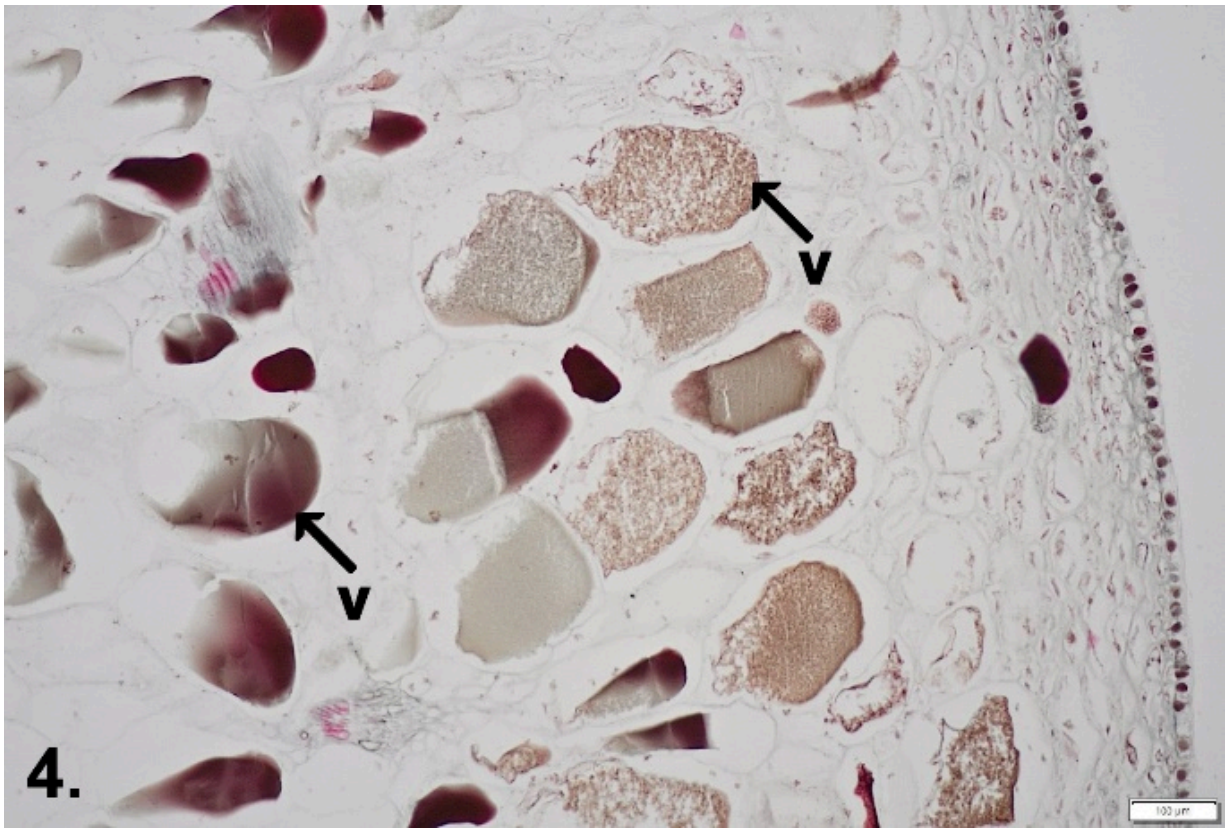

Figure 4

Cross-sections of *Prunus spinosa*fruits and its spontaneous hybrids. 1. *P.* ×*futicans* bar=10 μm, 2. *P. spinosa* bar=10 μm, 3. *P. spinosa* bar=20 μm, 4. *P.* ×*fruticans* bar= 100 μm, 5. P. ×*dominii*, bar=100 μm , 6. *P.* ×*dominii* bar=100 μm; ag – anthocyanis granules, c – cuticle, e - epidermis, h – hypodermis, w – wax

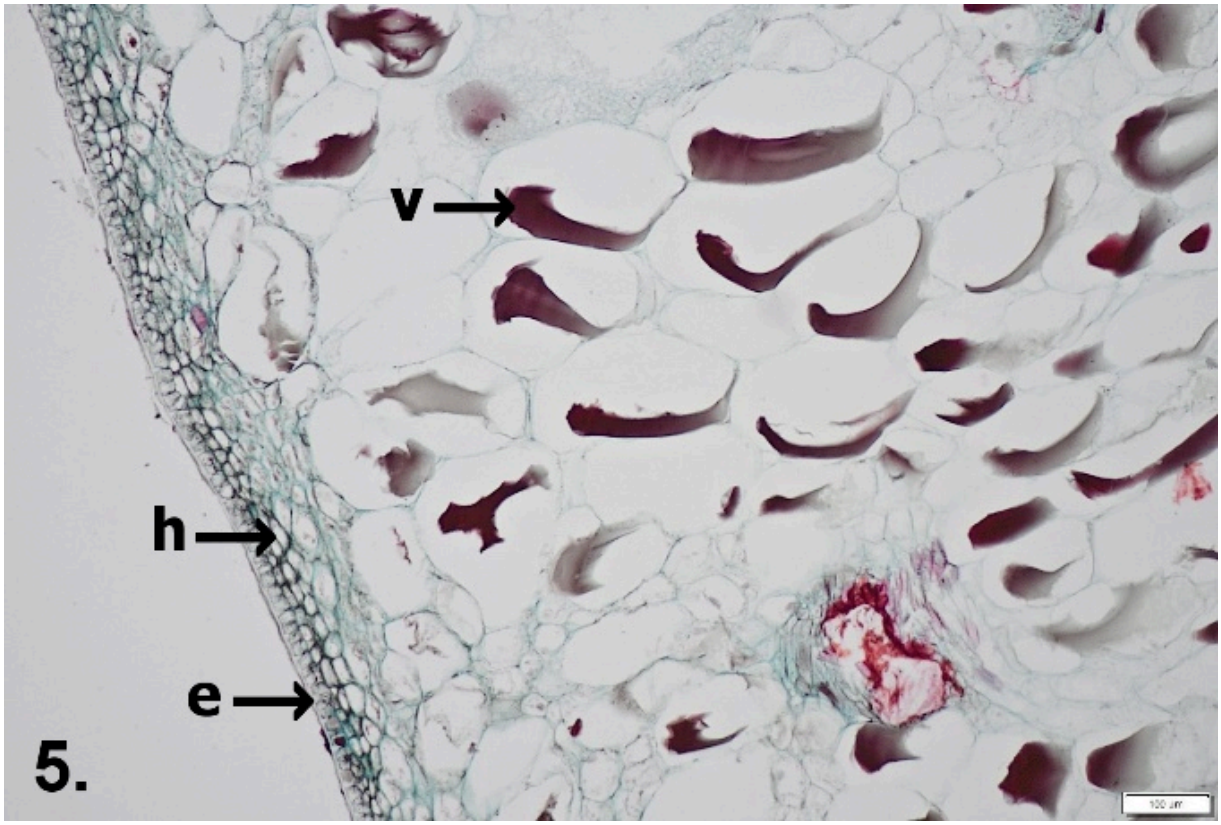

Figure 5

Cross-sections of *Prunus spinosa*fruits and its spontaneous hybrids. 1. *P.* ×*futicans* bar=10 µm, 2. *P. spinosa* bar=10 µm, 3. *P. spinosa* bar=20 µm, 4. *P.* ×*fruticans* bar= 100 µm, 5. P. ×*dominii*, bar=100 µm , 6. *P.* ×*dominii* bar=100 µm; ag – anthocyanis granules, c – cuticle, e - epidermis, h – hypodermis, w – wax

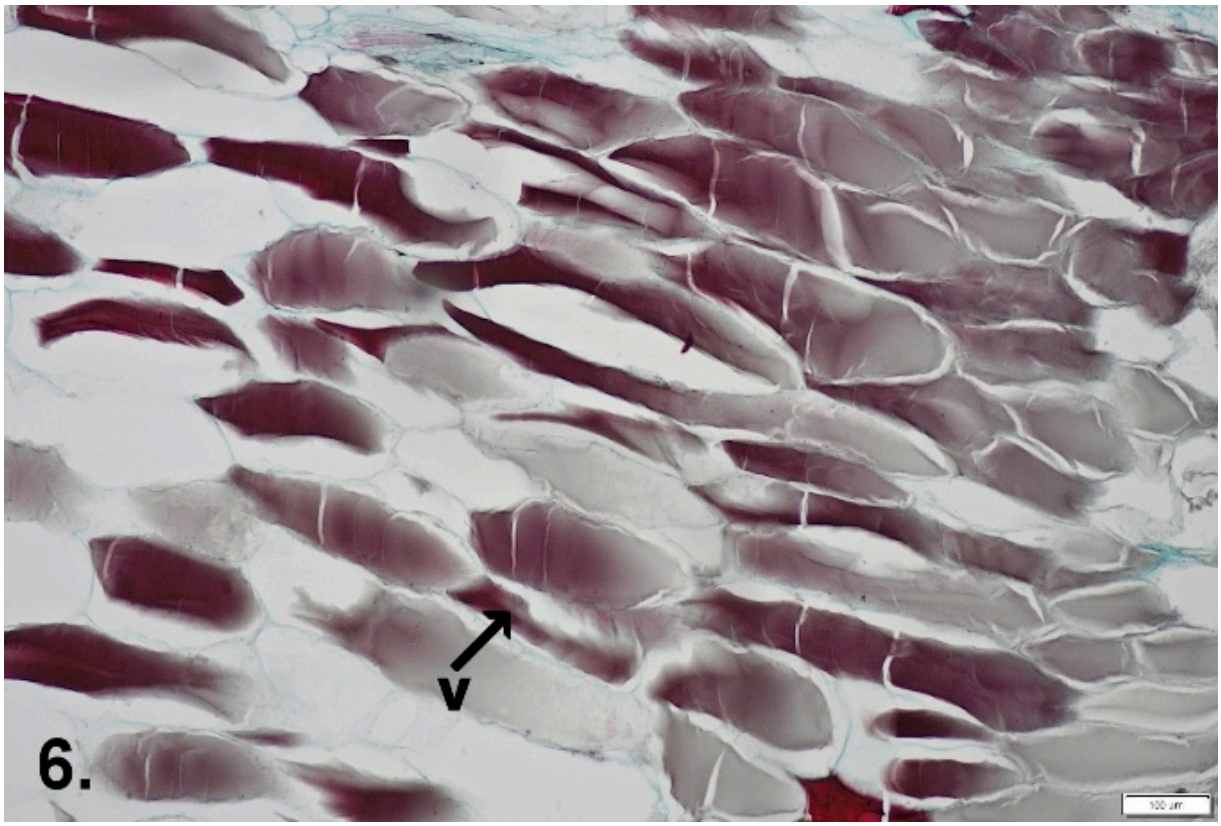

Figure 6

Cross-sections of *Prunus spinosa*fruits and its spontaneous hybrids. 1. *P.* ×*futicans* bar=10 µm, 2. *P. spinosa* bar=10 µm, 3. *P. spinosa* bar=20 µm, 4. *P.* ×*fruticans* bar= 100 µm, 5. P. ×*dominii*, bar=100 µm , 6. *P.* ×*dominii* bar=100 µm; ag – anthocyanis granules, c – cuticle, e - epidermis, h – hypodermis, w – wax