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Digestibility-related histological attributes of vegetative organs of barrel medic (*Medicago truncatula* Gaertn.) cultivars

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Abstract

An anatomical analysis of vegetative organs of barrel medic (*Medicago truncatula* Gaertn.) cultivars was performed using anatomical and stereological methods. The aim was to determine histological bases of the genotypic differences for digestibility, calculate tissue volume densities (*Vv*), examine the variability and assess tissue proportions along the stem maturity gradient. As the cultivars showed similar proportions of leaf tissues, potential variations in digestibility should be assigned to structural differences observed for the stems. Phloem and xylem *Vv* increased linearly with stem maturation, whilst *Vv* of epidermis, mechanical and parenchyma tissue significantly decreased. Secondary xylem was the tissue with the highest potential impact on digestibility. Cultivars 'Mogul' and 'Jemalong' had low proportion of lignified stem tissues, whilst 'Parabinga' and 'Caliph' did not show favourable anatomical characteristics.

More advantageous proportions of thick-walled and thin-walled cells were recorded in *M. truncatula* stems, compared to alfalfa (*M. sativa*). Therefore, examined cultivars are comparable in anatomical quality parameters to this well-known forage crop. Leaf parameters did not provide variability necessary for the improvement of forage quality. Stem parameters showed higher within-cultivar variability, which could be useful as a starting point in breeding towards improvement of digestibility. As the secondary xylem was the lignified stem tissue with the highest *Vv*, we recommend the improvement of *M. truncatula* digestibility through the reduction of its proportion.

Key words: anatomy, digestibility, Fabaceae, *Medicago truncatula,* stereology.

Introduction

Barrel medic (*Medicago truncatula* Gaertn.) is a model species for legume research, but also an important pasture species itself. It can be used for livestock feeding and soil improvement, as a pure stand or in companion cropping (Mihailović et al., 2011; Lagunes Espinoza, Julier, 2013). Preliminary research was conducted on the potential of *M. truncatula* growing for forage production in the agro-ecological conditions of northern Serbia (Mihailović et al., 2011). These authors provided morphological description of the cultivars (e.g., stem height, the number of stems, leaves, flowers and pods), as well as data about forage yield and dry matter chemical composition. Although commercial Australian varieties were often not well adapted to European environment (Porqueddu, 2001), the findings showed that eight tested cultivars of Australian origin produced adequate forage yields of relatively good quality, and, in some cases, lead to reliable seed productivity during a short vegetative season (Mihailović et al., 2011).

Schnurr et al. (2007) compared stem traits of one of the best forage crops, *M. truncatula*, which has a complex genetic structure, and *M. truncatula*, its close wild relative, a forage species with a small, diploid genome. They found that stem tissue morphology, cell wall lignification and tissue development were similar in these two species and concluded that *M. truncatula* could be used as a suitable model species for further *M. sativa* stem research. Concerning the proportion of tissues, their results showed that *M. sativa* stems were characterized by a higher proportion of xylem, whereas *M. truncatula* stems had larger phloem fibre bundles. Lagunes Espinoza and Julier (2013) confirmed tight relationship between quality and histology of *M. truncatula* stem. High plant digestibility was correlated to high stem digestibility and high cortex proportion. The structure and development of *M. truncatula* pod wall and seed coat was examined by Wang and Grusak (2005). More anatomical research was performed on *M. sativa*, highly economically important forage legume of the same genus, with the emphasis on its stem structure (Engels, Jung, 1998; Jung, Engels, 2002; Guines et al., 2003; Jung, Lamb, 2006; Zorić et al., 2012). Alfalfa stem anatomical characteristics, primarily the amount and distribution of lignified tissues, proved to be one of the most important factors that limit forage digestibility (Guines et al., 2001). Negative relationship was recorded between *in vitro* digestibility of alfalfa stems

and the area of lignified tissues in the stem cross-section (Guines et al., 2003), as well as lignin concentration (Jung, Allen, 1995). The digestibility decreases with plant maturity, due to increased cell wall proportion in stems, lignification and decreased leaf to stem ratio (Julier, Huyghe, 1997; Jung, Engels, 2002). Distribution of lignified tissues also affects the accessibility of polysaccharides to rumen microorganisms (Jung et al., 2000). Gronwald and Bucciarelli (2012) analysed stem anatomy of two alfalfa cultivars, and concluded that proportions of secondary xylem and pith parenchyma could not solely explain differences in lignin, cellulose and cell wall concentrations. Cetin (2009) found that excess of boron or its deficiency have negative effect on formation of alfalfa stem tissues, but least affect formation of xylem, although a significant reduction of its proportion was recorded.

The main tasks of forage breeders should be to reduce the amount of non-degradable barriers, particularly in stems, which are the main component of forage yield and determine the entire genotype digestibility (Guines et al., 2001). Legume leaves are characterized by high protein content, do not exhibit the increase in cell wall proportion associated with maturation and are more digestible than stems (Jung, Allen, 1995). Non-lignified tissues (collenchyma, chlorenchyma, cambium, phloem, and parenchyma) are more rapidly and almost completely degraded in animal digestive system, whilst lignified tissues, such as xylem vessels and phloem and xylem fibers, typically resist degradation (Engels, Jung, 2005; Jung, Lamb, 2006). Epidermal and collenchyma cells develop very thick, but not lignified primary cell walls, which are slowly degradable, whilst waxy cuticle tends to resist degradation (Jung, Engels, 2002). Differences between the cultivars in stem digestibility are usually influenced by variations in the number of vascular bundles or lignification of parenchyma cells between them (Porqueddu, 2001). In earlier studies, genotypes with high digestibility showed lower surface density of lignified cell walls along the full stem length, compared to genotypes with low digestibility. Variations in tissue types and the site and type of lignification were also related to digestibility in other legume genera (e.g., *Trifolium*, *Lathyrus*), as well as in grasses (Rezvani Moghaddam, Wilman, 1998; Wilman, Rezvani Moghaddam, 1998; Krstić et al., 2008; Zorić et al., 2011 a; b). As lignified tissues also have an important role in providing mechanical support and plant resistance to various biotic and abiotic factors, manipulation of proportions of these tissues is limited (Buxton, Redfearn, 1997).

We consider structural investigations and understanding of biological basis of forage crops particularly important, as these factors directly affect digestibility and nutritive value of forages (Krstić et al., 2008). Rinne et al. (2006) stated that a biological, rather than a chemical approach was needed in prediction of legume digestibility. In addition to classical anatomical approaches, stereological method for structural investigations of plant organs proved to be useful in the estimation of percentage of different tissues, and consequently prediction of forage quality (Zorić et al., 2011 a). This method enables estimation of tree-dimensional parameters, such as volumes of specific tissues, from the measurements available on several randomly sampled two-dimensional sections. It was of particular value in separate determination of volume density of intercellulars and more complete analysis of tissues from different angles, given that it enabled analysis of the plant organ as a whole, rather than just a segment. In this research we quantified histological characters of leaf blades and stems of eight cultivars, using stereological and classic anatomical method.

The aim was to determine histological bases of the genotypic differences that could explain variation for digestibility, calculate proportion of specific tissues in leaves and stems, examine the variability of anatomical characters and to assess tissue proportions along the stem maturity gradient.

Material and methods

The plants were grown in 2010 at the experimental field of the Institute of Field and Vegetable Crops at Rimski Šančevi, North Serbia (45°20′ N, 19°51′ E, 84 m a.s.l.). The analyses were performed in the Laboratory of Plant Anatomy and Morphology, University of Novi Sad, on eight barrel medic (*Medicago truncatula* Gaertn.) cultivars of Australian origin: 'Borung' (1), 'Caliph' (2), 'Jemalong' (3), 'Jester' (4), 'Mogul' (5), 'Parabinga' (6), 'Paraggio' (7) and 'Sephi' (8), provided by South Australian Research and Development Institute. The trial was established in April. The monthly temperature ranged from 13 to 23 °C (average 19°C) and monthly precipitation from 71 to 174 mm. The cultivars were sown at density of about 250 viable seeds per m^2, in seven rows occupying a plot of 1 m^2 area. The plants were cut in full bloom, while forming the first pods, thus a desirable balance between yield and forage quality was obtained in most of the legume forages. The plant material for histological analyses was fixed in 50% ethanol.

Ten plants of each cultivar (two from each inner row) were used for classical anatomical studies. Cross-sections were made at the middle part of the median lateral leaflet (at the main vein and at ¼ of the leaflet width), and at the middle part of its main stem. Five plants were used for stereological investigations, according to methodology developed by Kubinova (1991; 1993). Five to seven randomly chosen leaf tissue blocks were sampled from each leaf, using a sampling grid. The modified method for trifoliate leaves was applied (Zorić et al., 2011 a). In order to increase the level of unbiased sampling, leaf blocks were cut in four directions, instead of one (0º, 45º, 90º and 135º). Stem cross-sections were cut in positions sampled according to the principle of systematic uniform random sampling, along the stem axis (Kubinova, 1991), as shown in Figure 1. Based on the stem height, which was measured first, the interval (T) between the sampled sections (segments) was 3–8 cm, resulting in five sections per stem. The position of *z* of the first section was randomly selected within the interval 0–3 (8) cm and the segments were numbered incrementally, starting from the bottom of the stem.

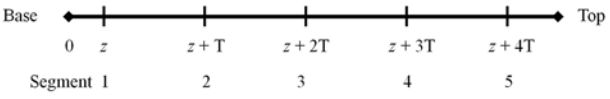


Figure 1. Systematic uniform random sampling of barrel medic (*Medicago truncatula*) stem segments along the stem axis for stereological analysis

All sections were obtained using a cryostat "Leica CM 1850" ("Leica", Germany), at a temperature of −20°C, at cutting intervals of 40 μm. Sections were stained for lignin using acid phloroglucinol test. Light microscopy observations and quantitative measurements of histological characters were made using light microscopy and image analyzing system "Motic Images Plus" ("Motic", Germany). The proportion of tissues was estimated by point-counting method, using the point grid test system of 192 test points. Approximately 1500–2000 points per leaf and 2000–2500 points per stem were counted. Based on the data obtained by stereological analysis, volume densities of tissues were calculated using the equation given by Kubinova (1993):

$$Vv(\mathrm{x}) = \frac{\sum_{j=1}^{n} Pj(\mathrm{x})}{\sum_{j=1}^{n} Pj(\mathrm{y})},$$

where n is the number of examined sections, Pj(x) (j = 1, ..., n) – number of test points hitting the specific tissue on j-number of sections and Pj(y) (j = 1, ... , n) – number of test points hitting the entire leaf cross section on j-number of sections.

Data were statistically processed and mean coefficients of variation and correlation coefficients were calculated using *STATISTICA* for *Windows*, version *10.0*. The significance of differences in measured parameters between the cultivars was determined using Duncan's test ($p \leq 0.05$). In order to establish the general structure of variability of anatomical parameters in eight genotypes, principal component analysis (PCA), based on correlation matrix, was applied.

Results and discussion

Lamina anatomical characteristics. Lamina was dorsiventral, comprising a single layer of epidermis, two layers of palisade and 4–5 layers of spongy tissue cells. Vascular bundles, surrounded with parenchyma sheath, were arranged in a single row. The main vein was prominent on abaxial side and contained one large vascular bundle. Solitary, prismatic crystals were present in bundle sheath cells. Volume densities (Vv) of lamina tissues were similar among the cultivars (Table 1). High level of variability was recorded for Vv of vascular and sclerenchyma tissues in each cultivar, although differences were not significant. This parameter was significantly negatively correlated with Vv of palisade tissue and intercellulars ($r = -0.495$ and -0.515, respectively).

In all analyzed cultivars, palisade/spongy tissue ratio, as one of the indicators of xeromorphic adaptations, was lower than 1 (Table 1). The highest value was recorded in 'Jester' and 'Borung', and the lowest in 'Mogul', which also had the smallest palisade and epidermal cells. 'Borung' and 'Jemalong' could be singled out as the cultivars with relatively large cells of lamina tissues. Examined cultivars showed similar values of the proportions of all lamina tissues. We believe that improvement of digestibility could not be reached by changes in lamina histology.

Table 1. Volume densities (Vv) of leaf tissues and leaf anatomical parameters of barrel medic (*Medicago truncatula*) cultivars (mean %, CV %)

Cultivar	Volume density (Vv)					
	epidermis	palisade cells	spongy cells	vascular tissue + sclerenchyma	parenchyma sheath	intercellulars in mesophyll
Borung	16.8 (10.3) ab*	25.5 (15.5) abc	27.6 (33.0) ab	8.8 (42.3) a	2.5 (49.7) ab	18.9 (35.6) ab
Caliph	17.7 (25.6) ab	30.6 (13.2) a	27.2 (8.3) ab	5.7 (9.7) a	2.5 (15.3) ab	16.3 (10.0) b
Jemalong	14.6 (7.5) b	30.4 (12.4) ab	23.6 (18.3) ab	4.9 (31.4) a	2.5 (44.9) ab	24.0 (18.3) a
Jester	18.4 (11.4) a	28.2 (12.5) abc	21.0 (8.0) b	9.0 (40.4) a	3.1 (43.8) ab	20.3 (13.2) ab
Mogul	17.4 (17.3) ab	25.1 (17.3) bc	27.9 (12.1) a	6.3 (34.1) a	4.0 (32.2) a	19.3 (20.6) ab
Parabinga	15.9 (4.2) ab	28.3 (10.7) abc	23.0 (19.8) ab	8.4 (33.8) a	2.4 (42.8) b	22.0 (17.3) ab
Paraggio	17.3 (17.7) ab	28.4 (7.1) abc	24.9 (10.7) ab	7.2 (46.0) a	2.2 (22.0) b	20.0 (25.7) ab
Sephi	19.4 (4.9) a	24.7 (15.3) c	22.4 (15.5) ab	8.7 (63.8) a	3.1 (37.5) ab	21.6 (9.0) ab
			Cell cross-section area μm²			
	Lamina thickness μm	Palisade/spongy tissue ratio	adaxial epidermal cells	abaxial epidermal cells	palisade cells	spongy cells
Borung	205 (12.1) ab*	0.87 (11.6) a	684 (23.0) bc	693 (19.8) ab	584 (16.4) a	309 (23.5) a
Caliph	180 (13.4) bc	0.83 (15.8) ab	616 (26.2) cde	622 (27.7) bc	490 (21.4) b	201 (25.7) def
Jemalong	208 (16.3) ab	0.70 (25.0) b	707 (24.0) b	733 (27.6) a	595 (19.6) a	228 (22.9) bcd
Jester	191 (11.7) abc	0.88 (10.3) a	557 (20.3) ef	618 (24.3) bc	477 (28.5) b	186 (21.3) ef
Mogul	207 (11.8) ab	0.54 (22.9) c	499 (27.3) f	584 (30.4) c	447 (24.9) b	255 (37.8) b
Parabinga	222 (15.7) a	0.70 (10.5) b	800 (24.1) a	754 (33.7) a	486 (25.2) b	242 (25.2) bc
Paraggio	186 (8.2) bc	0.74 (14.2) b	586 (20.7) de	544 (25.0) c	454 (30.0) b	173 (29.1) f
Sephi	171 (14.1) c	0.74 (10.2) b	659 (29.3) bcd	759 (26.8) a	462 (21.6) b	215 (22.2) cde

Note. * – the difference between the values with the same letter was not statistically significant between the cultivars at $p \leq 0.05$ according to Duncan's test.

Stem anatomical characteristics. The top part of the stem is an elongation zone, and is therefore the youngest and of primary structure. Cambial ring formation results in secondary stem growth, and leads to lignin deposition, especially in secondary walls of xylem tissue. Epidermal cells developed thick primary walls, especially the outer ones, covered with a waxy cuticle. In younger stem segments epidermis had higher Vv (~3%), compared to older segments (~2.5%) (Table 2, Fig. 2). Collenchyma cells were grouped in four longitudinal bundles, subepidermally. The cell wall thickening of these cells begins very early, and was clearly visible in the youngest segment, where their Vv was significantly the highest. These cells did not exhibit positive reaction to phloroglucinol, indicating the absence of lignin. The collenchyma Vv was significantly lower in more mature

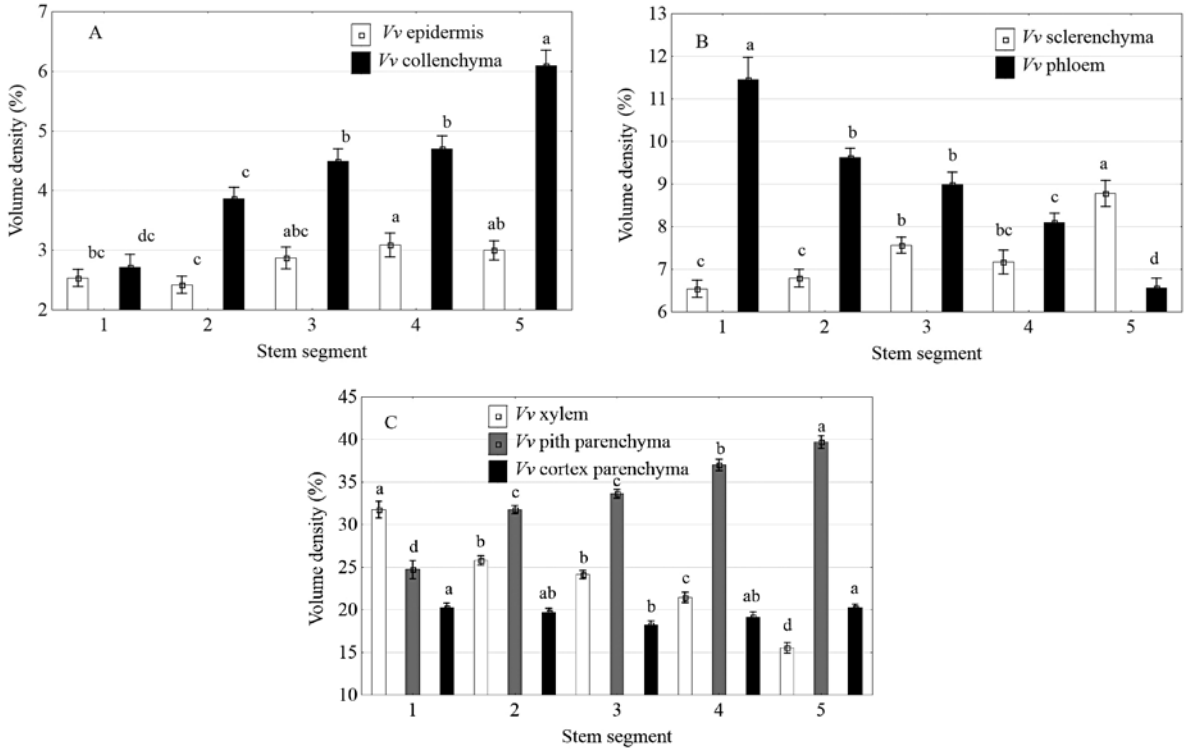
stem segments, as secondary thickening of the stem did not result in formation of new collenchyma cells (Figs 2 and 4). The groups of sclerenchyma cells along the primary phloem did not have lignified cell walls in the top part of the stems, where their *Vv* was significantly the highest (Figs 2–4). Lignification began in the second segment from the top. In older stem segments, sclerenchyma *Vv* values were significantly lower, down to 6.5% in the bottom segment, as even though stem continued with secondary thickening due to cambial activity, but new sclerenchyma elements were not formed.

Table 2. Volume densities (*Vv*) of stem tissues and stem anatomical parameters of barrel medic (*Medicago truncatula*) cultivars (mean %, CV %)

Cultivar	Volume density (*Vv*)						
	epidermis	collenchyma	cortex parenchyma	sclerenchyma	phloem	xylem	pith parenchyma
Borung	2.9 (43.4) abcd	4.4 (34.9) a	18.3 (12.8) cde	7.6 (17.6) abc	8.8 (23.9) bc	25.2 (22.1) ab	32.7 (17.0) ab
Caliph	2.6 (32.8) bcd	4.5 (25.0) a	17.4 (13.5) e	6.6 (20.1) c	8.1 (25.3) bc	26.3 (26.0) a	34.5 (18.8) ab
Jemalong	3.1 (25.5) ab	4.8 (28.4) a	19.5 (11.7) bcd	7.5 (22.2) bc	7.8 (20.8) c	22.0 (34.3) bc	35.3 (16.2) b
Jester	3.3 (35.4) a	3.0 (31.0) b	18.0 (11.7) de	7.0 (20.2) bc	8.8 (24.2) bc	23.5 (20.3) abc	36.3 (13.8) a
Mogul	2.3 (43.9) d	4.0 (58.1) ab	23.4 (14.4) a	8.5 (23.7) a	8.3 (25.2) bc	20.5 (25.7) c	34.0 (17.8) abc
Parabinga	3.0 (43.7) abc	4.7 (42.2) a	19.2 (12.9) bcd	6.7 (19.3) c	9.1 (19.1) bc	27.1 (19.4) a	30.1 (12.3) c
Paraggio	2.6 (35.3) bcd	4.9 (42.9) a	19.9 (19.0) bc	7.2 (24.3) bc	9.6 (31.9) b	24.0 (41.4) abc	31.8 (32.8) bc
Sephi	2.4 (40.9) cd	4.7 (37.3) a	20.4 (14.6) b	7.7 (25.6) ab	11.0 (36.0) a	21.4 (25.4) bc	32.3 (24.8) abc

			Cross-section area			
	No. of vascular bundles	Cortex thickness µm	stem mm^2	epidermal cells μm^2	cortex parenchyma cells μm^2	cylinder parenchyma cells μm^2
Borung	14.2 (5.9) a*	96.1 (16.6) ab	4.2 (11.7) a	406 (27.2) a	588 (21.1) a	11314 (22.8) ab
Caliph	14.2 (5.9) a	91.0 (5.3) b	3.9 (9.7) ab	225 (14.6) c	386 (21.5) d	9323 (25.6) cd
Jemalong	13.0 (10.9) ab	89.7 (14.2) b	3.6 (22.3) abc	360 (28.1) a	499 (23.8) b	11598 (26.5) a
Jester	13.6 (6.6) ab	64.6 (24.3) c	2.4 (14.5) d	297 (33.5) b	341 (27.0) d	7953 (25.7) def
Mogul	14.6 (15.8) a	112 (17.4) ab	2.9 (21.4) cd	217 (50.8) c	476 (30.3) b	8546 (25.6) de
Parabinga	11.0 (0.0) c	92.7 (21.1) b	2.7 (27.8) cd	288 (32.3) b	400 (36.0) cd	10045 (26.7) bc
Paraggio	12.4 (9.2) bc	106 (18.0) ab	3.0 (25.9) bcd	227 (29.0) c	366 (25.2) d	6929 (17.9) f
Sephi	12.4 (4.4) bc	118 (15.0) a	4.0 (21.8) a	277 (23.8) b	463 (36.7) bc	7759 (29.9) ef

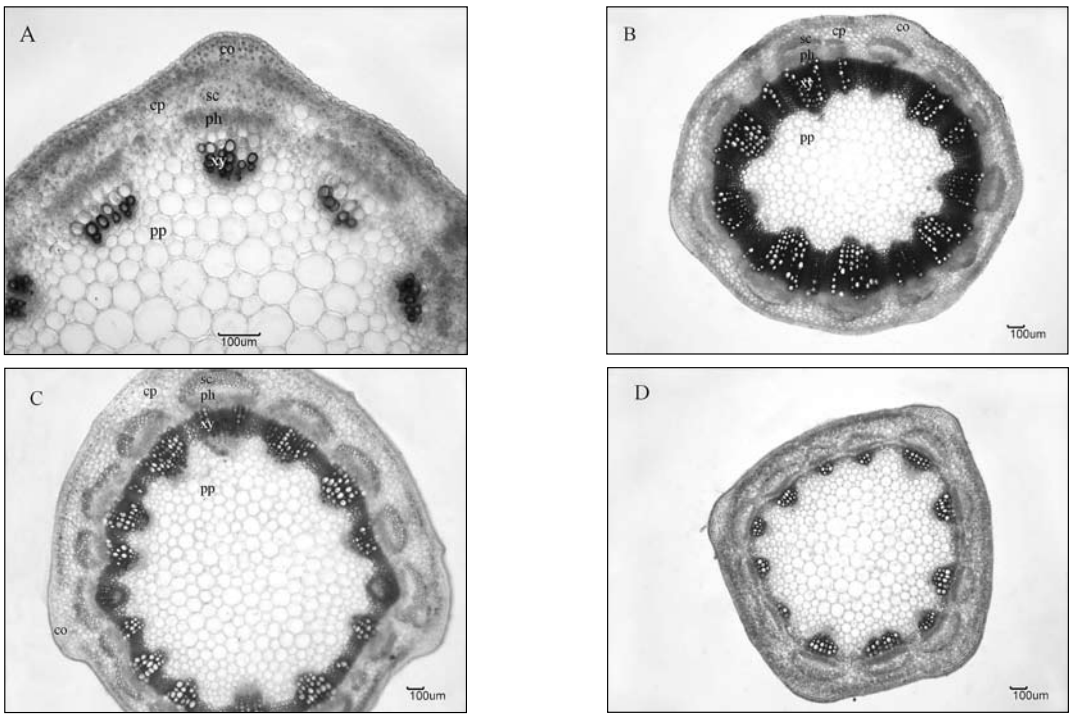
Note. * – the difference between the values with the same letter was not statistically significant between the cultivars at $p \leq 0.05$ according to Duncan's test.



Note. Letters indicate significance of differences between the segments, according to Duncan's test, $p < 0.05$ (mean, whisker: mean ± SE).

Figure 2. Volume densities (*Vv*) of stem tissues of different stem segments, averaged for all barrel medic (*Medicago truncatula*) cultivars (1 – bottom part, 5 – top part)

Cortex parenchyma is composed of assimilatory parenchyma, cortex parenchyma and parenchyma sheath cells, all of which have thin, non-lignified primary cell walls. The values of cortex parenchyma *Vv* did not vary significantly along the stem (Figs 2–4). Pith parenchyma cells in early stages of development were also characterized by thin, non-lignified cell walls along the full stem length. In more mature internodes, one or two layers of cells in perimedullar zone were slightly lignified. Pith parenchyma *Vv* decreased significantly with stem maturity, ranging from 39.7% at the top to 24.7% at the bottom of the stem.



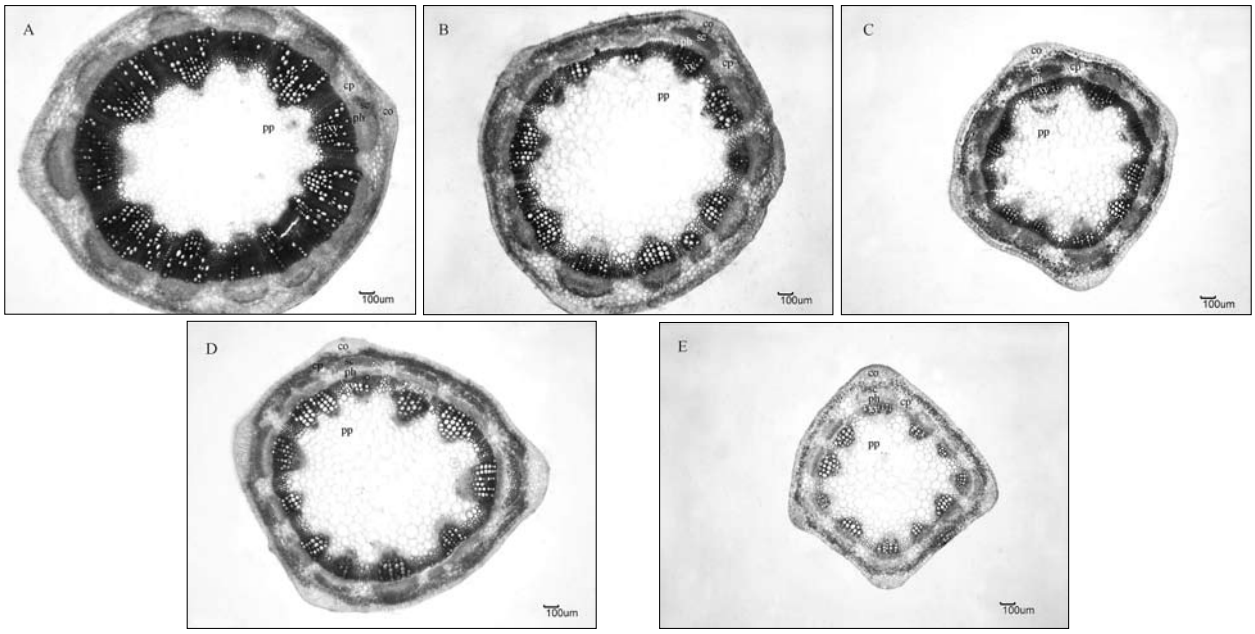
Notes. A – cultivar 'Jester', the youngest stem segment; primary xylem was the only lignified tissue. B – cultivar 'Jester', the oldest stem segment; strong lignification of secondary xylem and less lignified sclerenchyma tissue. C – cultivar 'Mogul', middle stem segment; characterized by favourable stem structural characteristics affecting digestibility, the lowest *Vv* of xylem and the highest *Vv* of cortex parenchyma. D – cultivar 'Jemalong', middle stem segment; favourable stem structural characteristics related to digestibility, low *Vv* of xylem and phloem and large epidermal and parenchyma cells. co – collenchyma, cp – cortex parenchyma, ph – phloem, pp – pith parenchyma, sc – sclerenchyma, xy – xylem.

Figure 3. Light micrographs of barrel medic (*Medicago truncatula*) stem cross-sections, stained for lignin using acid phloroglucinol test

Primary xylem was the only lignified tissue in the youngest, top part of the stems (Figs 2–4). However, in lower, fourth stem segment, cambial activity already resulted in the formation of secondary xylem, comprising lignified elements, as well as the secondary phloem. Percentage of xylem increased progressively from the apex to the basis of the stems. In the first segment, where xylem *Vv* was significantly the highest, lignification became very intensive. Phloem *Vv* also increased significantly with maturity of the stem segments, due to the intensive cambial activity (Figs 2 and 4). Correlation coefficients indicated significant negative correlations between *Vv* of xylem and *Vv* of collenchyma, sclerenchyma and parenchyma. Differences amongst the cultivars were not significant for a large majority of volume density parameters (Table 2). Cortex parenchyma *Vv* was significantly the highest in 'Mogul', but the weakest in 'Caliph', 'Jester' and 'Borung'. Sclerenchyma tissue was best developed in 'Mogul', whilst the lowest *Vv* were recorded in 'Caliph' and 'Parabinga'. The xylem tissue had the highest *Vv* in 'Parabinga' and 'Caliph', and the lowest in 'Mogul'. Phloem *Vv* was significantly the highest in 'Sephi' and the lowest in 'Jemalong', whilst other cultivars showed similar values. Values of pith parenchyma *Vv* were similar in all cultivars. The greatest stem cross-section area was measured in 'Borung', followed by 'Sephi' (Table 2). The average number of vascular bundles was the highest and exceeded 14 in 'Borung', 'Caliph' and 'Mogul', whilst in 'Parabinga' it was only 11. 'Jester' had the smallest stem cross-section and cortex thickness. Considering the size of the stem cells, 'Borung' and 'Jemalong' could be singled out as the cultivars with relatively large, whilst 'Paraggio', 'Jester' and 'Caliph' with relatively small cells.

Principal component analysis (PCA). The results of the PCA indicated that histological parameters had low variability and most of them did not contribute significantly to the total variation (Table 3). The first component explained only 18.99% of variation and was defined by the *Vv* of stem tissues. These parameters showed higher level of variability also due to the fact that they changed with stem maturity, from the bottom to the top stem part. Other characters, those of the leaves in particular, varied within a small range. Due to such low variability, the cultivars did not form any clusters and could not be separated or grouped using PCA analysis.

The results of PCA, which demonstrated that only stem histological parameters had higher and significant level of variability, also supported the claim that changes in lamina histology would not contribute to improvement of digestibility. Primary xylem was the only lignified tissue in the youngest part of the stem, which was related to its very important role in water conduction. The same was true for *M. sativa* stems,



A – segment 1, the bottom part of the stem, B – segment 2, C – segment 3, D – segment 4, E – segment 5, the top part of the stem; co – collenchyma, cp – cortex parenchyma, ph – phloem, pp – pith parenchyma, sc – sclerenchyma, xy – xylem

Figure 4. Light micrographs of cross-sections of barrel medic (*Medicago truncatula*) cv. 'Parabinga' stem segments (1–5), stained for lignin using acid phloroglucinol test

Table 3. Principal component analysis (PCA) of leaf and stem histological and anatomical parameters (only loadings significant for the axis presented, >0.700)

	Characters	PCA 1	PCA 2	PCA 3
Stem	*Vv* collenchyma	**0.756***	0.072	−0.093
	Vv sclerenchyma	**0.738***	−0.165	0.380
	Vv phloem	**−0.891***	−0.021	0.149
	Vv xylem	**−0.912***	0.155	−0.089
	Vv cylinder parenchyma	**0.902***	−0.113	−0.140
Lamina	cross-section area of adaxial epidermal cells	−0.016	**0.750***	−0.187
	% total variance explained	18.99	15.05	11.87

where lignification was first recorded in primary xylem, and later in secondary sclerenchyma (Vallet et al., 1996). Jung and Engels (2002) also reported that primary xylem was the only non-degraded tissue in immature *M. sativa* stems. According to Julier et al. (2008), the cell walls of xylem cells progressively increased, together with xylem thickness and pith parenchyma area, whilst cortex thickness remained constant from the top to the bottom part of *M. sativa* stem. Gronwald and Bucciarelli (2012) reported negative correlation between secondary xylem and pith proportion in *M. sativa* stem, because of an increasing cross-section area of secondary xylem due to cambial activity. Our results showed that *Vv* of phloem and xylem increased linearly from the top to the bottom of the stems, whilst *Vv* of epidermis, mechanical and parenchyma tissue significantly decreased. Secondary xylem proved to be the tissue with the strongest lignification and therefore had the highest potential impact on digestibility. The lignification of pith parenchyma cells in older stem parts, which begins simultaneously with intensive xylem development, is one more factor that could be associated with reduced digestibility. Knowledge of the critical point along the axis in percentage of lignified tissues is especially important in harvest management, as it helps determine optimal cutting height. Our results pointed to a significant increase of xylem *Vv* between $5^{th}/4^{th}$, $4^{th}/3^{rd}$ and $2^{nd}/1^{st}$ stem segments.

Among the examined cultivars, the most favourable ratio of lignified and non-lignified stem tissues was recorded in 'Mogul' and 'Jemalong'. Although 'Mogul' was the cultivar with the highest number of vascular bundles, it had the lowest *Vv* of xylem and the highest *Vv* of cortex parenchyma. 'Jemalong' also had low *Vv* of xylem, as well as large stem cells. According to Mihailovic et al. (2011), 'Mogul' and 'Jemalong' had stems with the highest number of branches per plant (7.1 and 6.9, respectively). 'Parabinga' with the highest *Vv* of xylem and the lowest *Vv* of parenchyma, and 'Caliph' with very high *Vv* of xylem, low *Vv* of cortex parenchyma and small stem cells, were the cultivars that did not exhibit favourable anatomical characteristics. According to the results of chemical analyses performed by Mihailović et al. (2011) on the same plant material, these two cultivars also had high lignin content and high neutral detergent fiber (NDF) content, which is in accordance with our results. They also had lower number of stem branches (3.2 and 4.9, respectively) and high forage dry matter proportion. As was expected, 'Jemalong' showed relatively low neutral detergent fiber and lignin content, which was not the case for 'Mogul'. However, these findings can be explained by the small size of 'Mogul' cells, especially

those in the stem, resulting in higher proportion of cell walls. Both 'Jemalong' and 'Mogul' had high herbage yield (30.7 and 29.3 t ha^{-1}, respectively), but low forage dry matter proportion (Mihailovic et al., 2011).

According to Gronwald and Bucciarelli (2012), the proportions of secondary xylem and pith parenchyma in stems of two *M. sativa* cultivars were 14–24% and 50–61%, respectively. The values obtained in our research are slightly higher for xylem (21–27%), but lower for pith parenchyma (30–36%). Rezvani Moghaddam and Wilman (1998) reported that proportions of thick-walled cells in leaf and stem cross-sections of *M. sativa* were 4.6% and 36.6%, whilst of thin-walled cells 78.0% and 59.9%, respectively. Investigation on the same subject was also performed by Ahmad and Wilman (2001), who obtained similar values (5.1% and 37.1% for thick-walled cells, and 77.7% and 59.0% for thin-walled cells in leaves and stems, respectively). Following the same classification of cells, our results showed that *M. truncatula* cultivars had generally larger percentage of thick-walled cells in leaves (4.9–9.0%), but similar in stems (33.0–38.5%), compared to *M. sativa*. Moreover, thin-walled cells were present in smaller percentage in *M. truncatula* leaves (50.2–60.3%), but in larger percentage in stems (63.1–69.7%). The examined cultivars followed similar distribution pattern of tissues as *M. sativa*. Since more favourable percentages were recorded in *M. truncatula* stems, which are more important than are leaves in limitation of digestibility, we suggest that examined cultivars were comparable in anatomical quality parameters with *M. sativa*. Similar conclusions were drawn by Lagunes Espinoza and Julier (2013).

The size of the cells could also have significant impact on digestibility. Large, thin-walled cells are easier to digest, contain high levels of cell solubles and have high voluntary intake (Rezvani Moghaddam, Wilman, 1998). 'Borung' and 'Jemalong' could be singled out as the cultivars with the largest leaf and stem cells, which is a favourable characteristic in determining digestibility. The smallest cells were present in 'Mogul' (stem) and 'Paraggio' (leaf and stem), which implied higher proportion of cell walls compared to soluble part of the cells.

We noted that leaf anatomical parameters of *M. truncatula* cultivars did not provide variability necessary for the improvement of forage quality through traditional breeding. However, stem parameters showed higher within-cultivar variability, which could be useful as a starting point in a breeding process towards improvement of digestibility. As secondary xylem was the lignified tissue with the highest *Vv* in stem, we recommend that the digestibility of *M. truncatula* could be improved by the reduction of its proportion.

Conclusions

1. The results of stereological and classic anatomical analyses of lamina and stem could be useful in prediction of forage quality of barrel medic (*Medicago truncatula* Gaertn.).

2. Changes in leaf anatomical parameters could not be used for the improvement of forage quality, since the cultivars showed low variation and similar volume densities (*Vv*) of lamina tissues.

3. Volume densities of phloem and xylem increased linearly along the stem maturity gradient, whilst *Vv* of epidermis, mechanical and parenchyma tissue significantly decreased. Data about lignification and distribution of tissues along the stem are applicable in harvest management.

4. Stem secondary xylem was the tissue with the strongest lignification and therefore with the highest potential impact on digestibility. We suggest the improvement of *M. truncatula* digestibility through the reduction of its proportion.

5. Cultivars 'Mogul' and 'Jemalong' could be singled out as those with most favourable ratio of lignified and non-lignified tissues.

6. Examined *M. truncatula* cultivars are similar in anatomical quality parameters to one of the agronomically most important forage crops, alfalfa (*M. sativa*). These results contribute to the prediction that *M. truncatula* has significant potential for high quality forage production in Serbia.

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Bukosios liucernos (*Medicago truncatula* Gaertn.) veislių virškinamumui svarbūs vegetatyvinių organų histologiniai požymiai

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Santrauka

Bukosios liucernos (*Medicago truncatula* Gaertn.) veislių vegetatyvinių organų anatominė analizė buvo atlikta taikant audinių pjūvių ir paviršiaus tyrimo (stereologijos) metodus. Siekta histologiškai įvertinti genotipų virškinamumo skirtumus, apskaičiuoti audinių tūrio tankį (*Vv*), ištirti kintamumą ir audinių santykį išilgai stiebo nevienodai subrendusiose dalyse. Kadangi bukosios liucernos veislių lapų audinių santykiai buvo panašūs, virškinamumo variaciją galima paaiškinti stiebo sandaros skirtumais. Subrendusiose stiebo dalyse floemos ir ksilemos tūrio tankis buvo didesnis, o epidermio, mechaninės ir parenchimos audinio – mažesnis. Virškinamumui didžiausios įtakos turėjo antrinė ksilema. Veislės 'Mogul' ir 'Jemalong' pasižymėjo negausiais lignifikuotais audiniais, o veislėms 'Parabinga' ir 'Caliph' šios vertingos savybės nebuvo būdingos.

Palankesnis storasienių ir plonasienių ląstelių santykis buvo nustatytas bukosios liucernos stiebuose, lyginant su mėlynžiedės liucernos stiebais. Todėl galima teigti, kad pagal anatominius rodiklius bukosios liucernos veislės buvo panašios į gerai žinomos mėlynžiedės liucernos. Lapų rodiklių variacija nebuvo pakankama pašaro kokybei pagerinti. Stiebų audinių rodikliai labai įvairavo veislės viduje, ir jie galėtų būti panaudoti pradiniuose selekcijos etapuose, siekiant pagerinti liucernos virškinamumą. Kadangi stiebo lignifikuota antrinė ksilema pasižymėjo didžiausiu tūrio tankiu, bukosios liucernos virškinamumą būtų galima pagerinti mažinant santykinę šio audinio dalį tarp kitų stiebo audinių.

Reikšminiai žodžiai: anatomija, *Medicago truncatula*, pupiniai, stereologija, virškinamumas.