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The effect of field pea (*Pisum sativum* L.) as companion crop on leaf histological parameters of lucerne (*Medicago sativa* L.)

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Abstract

In this work, structural modifications of lucerne lamina were examined using light microscopy, in order to evaluate the effect of cover crop pea cultivar and sowing density on lamina anatomy. The plants were grown under companion cropping conditions with two field pea cultivars (Jezero - afila type, and Javor – type with small leaflets), at three cover crop sowing densities (30, 60 and 90 plants/m^2). The results showed that companion cropping did not significantly affect the lamina structure, proportion of lamina tissues or the size of the cells. All treatments showed low variability of measured parameters and high level of homogeneity, which was confirmed by PCA analysis. Heliomorphic parameters, such as higher palisade/spongy tissue ratio, larger palisade cells and thicker epidermis, were more expressed in control group plants and those grown under lower cover crop density. As both cover crop cultivars, at three applied sowing densities, transmitted sufficient sunlight to lucerne, companion growing did not negatively affect the leaf photosynthetic tissue. Our anatomical results indicate a potential for development of a new, reliable and environmentally friendly method of the lucerne establishment, without negative effect on the process of photosynthesis.

Keywords: intercropping, leaf anatomy, lucerne, pea, photosynthetic tissue.
Abbreviations: MDA – Multivariate Discriminant Function Analysis, PCA – Principal Component Analysis, SE – standard errors.

Introduction

The companion cropping with perennial legumes is deemed an effective method of agricultural and, more specifically, forage production, as it offers increased yield stability, higher yields, reduced weed competition, increased protein content within a mixed diet and higher land-use efficiency (Anil et al., 1998). Despite its advantages, the agricultural intensification in terms of plant breeding, mechanization, fertilizer and pesticide use experienced during the last 50 years has led to elimination of intercropping from many farming systems. The success of companion cropping depends on the capacity of the undersown crop to develop in the shade of the cover crop, as the competition for light, nutrients, and water may reduce the yield and resistance of the undersown crop, in particular when the grains used for planting are small (Tan et al., 2004). In the West Balkan Countries and beyond, however, it is small grains — primarily oats and barley — that are traditionally intercropped with perennial legumes. Given that these species tend to be too fast-growing, they are typically too competitive for the legume component to thrive. Information on alternative companion crops is limited (Sule, 1993).

Field pea (*Pisum sativum* L.) could be suitable for intercropping with lucerne (*Medicago sativa* L.) because N_2 fixation is improved, the crop can be harvested quickly and the canopy structure is not overly dense to cause suppressive shading (Cupina et al., 2011; Makoi and Ndakidemi, 2011). Modern field pea cultivars differ in morphology, primarily in leaf structure and plant height. The pea cultivars with short stems and leaflets reduced into tendrils — known as semi-leafless or afila type — are important for intercropping, as light penetration is much better, providing better conditions for the initial growth of the undersown crop (Koivisto, 2002). According to Simmons et al. (1995), the light intensity at the level of the perennial legume within the semi-dwarf companion crop canopy was consistently higher, compared to the conditions provided by conventional-stature companion crops. Moreover, the semi-leafless pea cultivars are able to convert solar radiation into dry matter more effectively than normal-leafed cultivars can (Heath and Hebblethwaite, 1985). In addition to the selection of a suitable field pea cultivar as the companion crop, an appropriate production technology also needs to be developed. To mitigate the effect of competition among the intercropped plants, reduction to the normal seeding rate of the companion crop is recommended. Hence the optimum stand density — i.e., the number of plants of the companion crop per unit area —needs to be determined (Vough et al., 1995; Cupina et al. 2010). Of all plant organs, the leaf is the most susceptible to the environmental factors, especially to the level of illumination and the amount of available water. Within the same individual plant, leaves developed in bright light conditions (sun leaves) tend to be smaller and thicker, with increased mesophyll tissue area, stronger, better developed mechanical tissue, and higher density of stomata and veins, compared to the leaves exposed to shade (shade leaves) (Dickison, 2000). It has been suggested that the strong light induces the

elongation of palisade cells. In leaves exposed to direct sunlight, the number of palisade layers is increased, the cells are long, large and close together, whilst in leaves in shade they are mostly short, thin and loosely arranged. The same is true for the spongy tissue cells; thus, the total volume of intercellular space may be twice as great in shade leaf as in a sun leaf of the same species (Arora and Gupta, 1996). In *M. sativa* leaves, the light microenvironment, leaf anatomy and photosynthesis seem to be strongly interrelated (Vogelmann et al., 1989). As light enters the leaf, the light gradient that forms within depends on the cell size and shape, organization, pigmentation and distribution of intercellulars inside the mesophyll (Martin et al., 1989). According to ecological indicator values given by Borhidi (1995), *M. sativa* is a full light species of open habitats, with maximum relative light indicator value of 9 out of 9, and the species that prefers habitats of relatively low humidity (with relative moisture indicator value 5 out of 12). This implies that this species demands high level of light exposure, and that growing in companion cropping might induce some unfavourable micro-ecological light conditions. However, as companion cropping with other agronomical important species could be more economically viable, it became necessary to investigate the effect of companion cropping on lucerne vegetative organs and forage quality. In the view of the above, the leaves of lucerne grown in companion cropping under two field pea cultivars differing in leaf morphology and hence in light interception capacity were examined and the effect of cover crop density evaluated. The objective was to assess the structural modifications and changes in proportional participation of specific tissue types in the overall leaf composition, with special emphasis on photosynthetic tissue.

Results

Lamina anatomical characteristics

Anatomical analysis showed that general structure of leaflet lamina did not differ between lucerne plants grown under different field pea population densities. All of the analyzed samples had dorsiventral lamina, with one layer of epidermal cells and well-defined palisade and spongy tissue (Fig. 1). Quantitative differences between the samples were recorded, in particular in the thickness of tissues and the size of epidermal and mesophyll cells: however, most of these differences were not statistically significant.

The effect of cultivar and cover crop density on lamina anatomy

The lucerne plants grown as a pure stand had significantly smaller and thinner laminas, higher percentage of epidermis, and higher palisade/spongy tissue ratio compared to the plants grown in companion cropping (Table 1 and 2). The same differences were recorded between the plants from the first and second control group. The effect of the cultivar, Jezero and Javor, as cover crop was statistically significant for lamina cross-section area, percentage of abaxial epidermis and the size of palisade tissue cells. Surprisingly, palisade tissue was better developed in lucerne plants grown under cultivar Javor.

The main effect of cover crop density was not significant for most of the analyzed parameters (Table 1 and 2). The plants grown under 30 cover crop plants per m^2 had significantly thicker palisade tissue and higher palisade/spongy tissue ratio compared to the plants grown under 60 or 90 cover crop plants per m^2. These plants also had somewhat smaller epidermal cells. All these characteristics are typical for plants exposed to bright light conditions and point to the fact that plants subjected to this treatment received more sunlight, than did plants grown under denser cover crop. Control plants differed significantly from those subjected to treatments in thinner lamina and spongy tissue, larger palisade cells and palisade/spongy tissue ratio and thicker epidermis. Significant differences were not recorded in most of the anatomical parameters amongst the lucerne plants grown under different densities of cultivar Jezero (Table 1 and 2). Those grown under 60 plants/m^2 had weaker palisade tissue, whereas those grown under lower density of cultivar Javor (30 plants/m^2) had significantly higher percentage of palisade tissue and smaller epidermal cells compared to plants grown under higher densities of the same cultivar (Table 1 and 2).

Principal Components Analysis of lamina anatomical parameters

The results of the Principal Components Analysis (PCA) is pointed to low variability in the analyzed lamina anatomical parameters (Table 3). The first principal component explained 33.02 % of total variability, with lamina thickness, percentage of epidermis and mesophyll, and the size of palisade cells as the predominant contributors. The second principal component, which contributed to total variability with 22.43 %, was defined by the palisade/spongy tissue ratio and percentage of spongy tissue. According to the type of variability, examined samples could not be grouped by PCA, as all of them showed similar type of variation and a high level of homogeneity.

Multivariate Discriminant Analysis

Although analyzed lucerne plants were anatomically very similar, Multivariate Discriminant Analysis showed that plants of the control group I (pure lucerne crop) differed from all other analyzed plant groups, and were separated on the first discriminant axis (Table 4, Fig. 2). Parameters that contributed the most to this discrimination were the smallest lamina cross-section area, high number of lamina vascular bundles, and high palisade/spongy tissue ratio. The percentages of mesophyll, palisade and spongy tissue also loaded heavily on the first axis, contributing more to discrimination along the second axis. Second discriminant axis further separated lucerne plants into two groups — those grown under cultivar Jezero and cultivar Javor as cover crops, respectively. This separation was based on the differences in percentage of mesophyll, palisade and spongy tissue, and the size of palisade cells. The plants from the control group II, grown with oat as a cover crop, were more similar to plants grown under Jezero cover crop.

Discussion

Lucerne grown under companion cropping establishment, as an alternative to growing as a pure crop, could be subjected to changes in micro-ecological growing conditions, in particular the amount of available sunlight. Shade-grown plants usually show some anatomical modifications of vegetative organs, especially leaves (Arora and Gupta, 1996; Dickison, 2000). Leaf structural adjustments to differences in light availability affect mostly photosynthetic apparatus. Although *M. sativa* was characterized as a species with high light requirements, our analyses showed that companion cropping did not significantly affect most of the parameters

Table 1. Lamina anatomical parameters of lucerne (means, standard errors and significance of differences between the treatments).

Variable		Cross-section area (mm^2)	Lamina thickness (µm)	Number of vasc. bundles	Cell cross-section area ($µm^2$)			
					Adaxial epidermis	Abaxial epidermis	Palisade tissue	Spongy tissue
Pure lucerne		1.2 ^{b*}	157.5 ^{b}	12.7 ^{a}	605 ^{a}	566 ^{a}	561 ^{a}	212 ^{a}
Cover crop		1.7 ^{a}	171.3 ^{a}	12.1 ^{a}	527 ^{b}	533 ^{a}	476 ^{a}	228 ^{a}
SE		0.07	2.48	0.19	14.5	14.3	25.5	4.7
Pure lucerne		1.2 ^{b}	157.5 ^{b}	12.7 ^{a}	605 ^{a}	566 ^{a}	561 ^{a}	212 ^{b}
Control - oat		1.7 ^{a}	174.1 ^{a}	12.3 ^{a}	565 ^{a}	517 ^{a}	416 ^{b}	194 ^{ab}
Pea cover crop		1.7 ^{a}	170.9 ^{a}	12.1 ^{a}	521 ^{b}	536 ^{a}	486 ^{ab}	233 ^{a}
SE		0.05	2.77	0.14	11.2	11.8	20.5	5.1
Pure lucerne		1.2 ^{c}	157.5 ^{b}	12.7 ^{a}	605 ^{a}	566 ^{a}	561 ^{a}	212 ^{b}
Jezero		1.5 ^{b}	168.8 ^{a}	12.1 ^{a}	521 ^{b}	569 ^{a}	414 ^{b}	226 ^{ab}
Javor		1.8 ^{a}	172.9 ^{a}	12.1 ^{a}	521 ^{b}	503 ^{a}	559 ^{a}	240 ^{a}
SE		0.06	1.84	0.14	12.1	16.8	22.7	5.2
Pure lucerne		1.2 ^{b}	157.5 ^{b}	12.7 ^{a}	605 ^{a}	566 ^{ab}	561 ^{a}	212 ^{b}
30 plants m^{-2}		1.6 ^{a}	171.7 ^{a}	12.6 ^{a}	475 ^{c}	482 ^{b}	507 ^{ab}	213 ^{b}
60 plants m^{-2}		1.7 ^{a}	169.7 ^{a}	12.4 ^{a}	524 ^{bc}	604 ^{a}	476 ^{b}	229 ^{b}
90 plants m^{-2}		1.6 ^{a}	171.2 ^{a}	11.3 ^{b}	564 ^{ab}	522 ^{ab}	476 ^{b}	259 ^{a}
SE		0.04	1.54	0.16	13.0	18.1	14.2	5.6
	30	1.4 ^{b}	168.6 ^{ab}	12.7 ^{a}	535.72 ^{a}	497 ^{b}	435 ^{a}	192 ^{a}
Jezero	60	1.7 ^{a}	173.9 ^{a}	13.2 ^{a}	516.15 ^{a}	736 ^{a}	438 ^{a}	239 ^{a}
	90	1.4 ^{b}	164.0 ^{b}	10.5 ^{b}	510.45 ^{a}	475 ^{b}	368 ^{a}	246 ^{a}
SE		0.03	1.68	0.32	16.477	48.2	18.2	12.2
	30	1.8 ^{a}	174.8 ^{a}	12.5 ^{a}	413.38 ^{c}	467 ^{b}	578 ^{a}	233 ^{b}
Javor	60	1.7 ^{b}	165.4 ^{b}	11.7 ^{a}	531.15 ^{b}	471 ^{b}	514 ^{b}	218 ^{b}
	90	1.9 ^{a}	178.4 ^{a}	12.2 ^{a}	617.27 ^{a}	570 ^{a}	584 ^{a}	270 ^{a}
SE		0.03	1.77	0.18	21.129	12.6	9.3	6.3

* - The difference between the values with the same letter was not statistically significant between the treatments at $p \leq 0.05$.

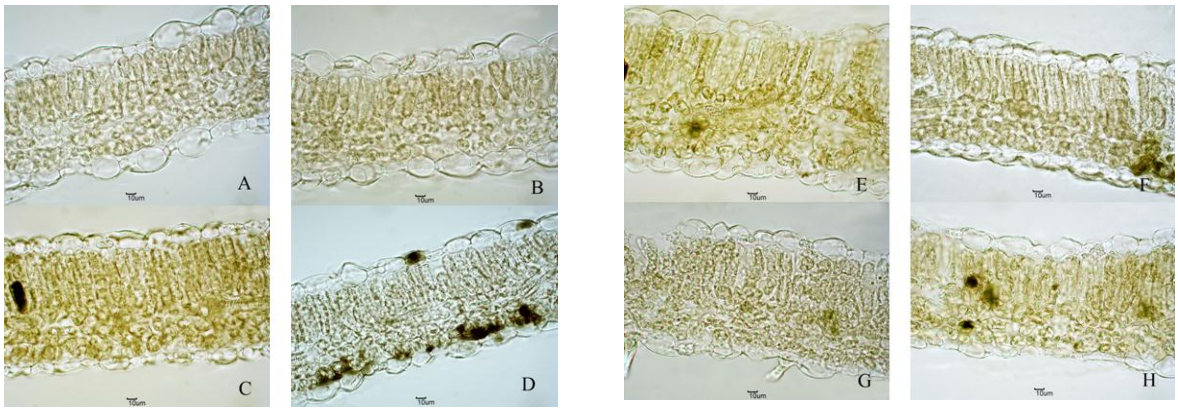

Fig 1. Cross-sections of lucerne lamina. Control I (A); Control II (B); plants grown in companion cropping with cultivar Jezero (C, E, G) and Javor (D, F, H); plants grown under 30 cover crop plants /m^2 (C, D), 60 cover crop plants /m^2 (E, F) and 90 cover crop plants /m^2 (G, H).

of the lamina structure, proportion of lamina tissues or the size of the cells. The plants from the control group had thicker epidermis and larger palisade cells compared to the plants grown in companion cropping, which could have been induced by higher illumination. The plants subjected to all treatments showed low variability of measured parameters and high level of homogeneity, which was confirmed by PCA. The cover crop cultivar (Javor or Jezero) as a factor did not induce significant differences in analyzed lucerne lamina anatomical parameters, as would be expected given the differences in cultivars morphology. Both cultivars transmit enough light to lucerne leaves, even though lucerne plants grown under Javor (with reduced leaflets) had better developed palisade tissue. Moreover, cover crop sowing density also did not show significant effect on the lucerne lamina anatomy. Heliomorphic parameters—stronger developed palisade tissue, higher palisade/spongy tissue ratio, and smaller epidermal and spongy tissue cells—were somewhat more expressed in plants grown under 30 cover crop plants/m^2. Although these differences were not always statistically significant, they indicated higher light availability for lucerne plants when grown under lower cover crop density. Afila pea cultivar Jezero made less shade to the lucerne plants grown underneath. Therefore, heliomorphism was more prominent in lucerne plants grown under lower cover crop sowing density with cultivar Javor as a cover crop. This might be explained by the fact that plants of this cultivar had leaves, and their higher sowing density increased the number of leaves covering lucerne plants, thus further reducing the amount of available light. The environmentally-induced anatomical variation may have significant consequences for photosynthesis. The better development of

Table 2. Lamina tissue percentages of lucerne (means, standard errors and significance of differences between the treatments).

Variable		Tissue, as a percentage of lamina thickness (%)					Palisade/ spongy tissue ratio
		Adaxial epidermis	Abaxial epidermis	Mesophyll	Palisade tissue	Spongy tissue	
Pure lucerne		13.5 ^{a*}	14.6 ^{a}	71.9 ^{b}	38.5 ^{a}	33.3 ^{b}	1.3 ^{a}
Cover crop		12.4 ^{a}	12.3 ^{b}	75.2 ^{a}	39.1 ^{a}	36.1 ^{a}	1.1 ^{b}
SE		0.25	0.37	0.82	0.44	0.55	0.03
Pure lucerne		13.5 ^{a}	14.6 ^{a}	71.9 ^{b}	38.5 ^{a}	33.3 ^{b}	1.3 ^{a}
Control - oat		12.7 ^{ab}	12.5 ^{b}	74.8 ^{ab}	40.0 ^{a}	34.8 ^{ab}	1.2 ^{ab}
Pea cover crop		12.4 ^{b}	12.3 ^{b}	75.3 ^{a}	39.0 ^{a}	36.3 ^{a}	1.1 ^{b}
SE		0.23	0.29	0.60	0.35	0.41	0.03
Pure lucerne		13.5 ^{a}	14.6 ^{a}	71.9 ^{b}	38.5 ^{b}	33.3 ^{b}	1.3 ^{a}
Jezero		12.5 ^{b}	12.9 ^{b}	74.5 ^{ab}	37.6 ^{b}	37.0 ^{a}	1.0 ^{c}
Javor		12.2 ^{b}	11.7 ^{c}	76.0 ^{a}	40.4 ^{a}	35.6 ^{a}	1.1 ^{b}
SE		0.19	0.32	0.69	0.46	0.47	0.03
Pure lucerne		13.5 ^{a}	14.6 ^{a}	71.9 ^{b}	38.5 ^{b}	33.3 ^{c}	1.3 ^{a}
30 plants m^{-2}		11.7 ^{c}	12.2 ^{b}	76.1 ^{a}	41.1 ^{a}	35.0 ^{b}	1.2 ^{a}
60 plants m^{-2}		12.6 ^{b}	12.1 ^{b}	75.3 ^{a}	37.9 ^{b}	37.4 ^{a}	1.0 ^{b}
90 plants m^{-2}		12.7 ^{b}	12.6 ^{b}	74.4 ^{ab}	38.0 ^{b}	36.5 ^{ab}	1.1 ^{b}
SE		0.18	0.24	0.52	0.40	0.41	0.02
Jezero	30	12.3 ^{a}	12.8 ^{a}	74.9 ^{a}	38.6 ^{a}	36.4 ^{a}	1.1 ^{a}
	60	12.5 ^{a}	13.1 ^{a}	74.3 ^{a}	36.4 ^{b}	37.9 ^{a}	1.0 ^{b}
	90	12.8 ^{a}	12.8 ^{a}	74.4 ^{a}	37.8 ^{ab}	36.6 ^{a}	1.1 ^{a}
SE		0.21	0.28	0.49	0.43	0.53	0.02
Javor	30	11.2 ^{b}	11.6 ^{ab}	77.2 ^{a}	43.6 ^{a}	33.6 ^{b}	1.3 ^{a}
	60	12.7 ^{a}	11.0 ^{b}	76.2 ^{a}	39.4 ^{b}	36.8 ^{a}	1.1 ^{b}
	90	12.7 ^{a}	12.3 ^{a}	74.5 ^{a}	38.1 ^{b}	36.4 ^{a}	1.1 ^{b}
SE		0.22	0.19	0.88	0.73	0.58	0.03

* - The difference between the values with the same letter was not statistically significant between the treatments at $p \leq 0.05$.

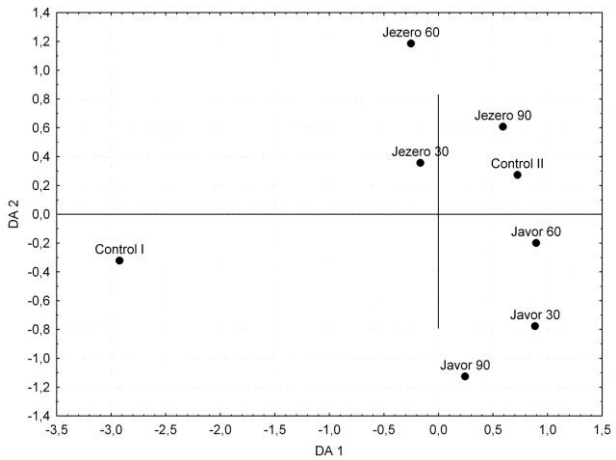

Fig 2. The results of the Multivariate Discriminant Analysis, projection of the first two factors. The first axis clearly discriminates control group I; the second axis discriminates lucerne plants grown under cultivar Javor and Jezero as a cover crop.

palisade mesophyll in sun leaves is positively correlated with photosynthetic capacity (Dickison, 2000). Compared to the control plants, those grown in companion cropping did not differ significantly in most of the lamina anatomical parameters. However, the combination of anatomical characteristics and type of their variability enabled clear differentiation of lucerne plants of the control group from all other plants, along the first discriminant axis of Multivariate Discriminant Analysis. This could be explained by the fact that these plants were grown as pure crops, under optimum light conditions. Consequently; their leaves exhibited typical sun leaf structure—smaller size, high palisade/spongy tissue ratio, higher number of vascular bundles and more mechanical tissue. These anatomical parameters, together with percentages of mesophyll, palisade and spongy tissue, defined the first discriminant axis. Further separation of lucerne plants into two groups, along the second discriminant axis, corresponded to their different growing conditions. Plants grown under cultivar Jezero were clearly separated from those grown under cultivar Javor, mainly based on percentages of mesophyll, palisade and spongy tissue. Those findings point to the conclusion that, although differences between the treatments were not statistically significant, two analyzed cultivars do create different growing conditions for lucerne plants underneath. Our findings demonstrate that companion cropping of lucerne with pea cultivars did not significantly affect its lamina structure. More importantly, it did not reduce the amount of received sunlight, which would

Table 3. Principal component analysis (PCA) of measured parameters. Factor coordinates of variables, based on correlations and cumulative percentages of the vectors (marked loadings are > 0.7000).

Characters	Factor 1	Factor 2	Factor 3	Factor 4
Lamina cross section area	-0.512	-0.327	0.616	0.234
Lamina thickness	-0.753*	-0.329	0.290	-0.132
Number of vasc. bundles	-0.089	-0.222	0.770*	0.042
Tissue, as a percentage of lamina thickness				
Adaxial epidermis	0.786*	0.082	0.273	0.345
Abaxial epidermis	0.839*	0.060	0.155	-0.108
Mesophyll	-0.929*	-0.066	-0.275	-0.135
Palisade tissue	-0.673	0.698	0.172	-0.053
Spongy tissue	-0.165	-0.848*	-0.462	-0.070
Palisade/spongy tissue ratio	-0.265	0.883*	0.327	0.014
Cell cross-section area				
Adaxial epidermis	0.274	-0.535	0.337	-0.018
Abaxial epidermis	0.284	-0.450	0.437	-0.513
Palisade tissue	-0.707*	-0.198	0.148	-0.018
Spongy tissue	-0.249	-0.352	-0.064	0.759*
Percentages of the vectors	33.02	22.43	14.70	8.23

Table 4. Multivariate Discriminant Analysis (MDA), standardized coefficients for canonical variables (marked loadings are > 0.7000 and significant for the axis).

Characters	Root 1	Root 2	Root 3	Root 4
Lamina cross section area	1.153*	-0.128	-0.432	0.303
Lamina thickness	0.179	0.857	0.882*	0.267
Number of vasc. bundles	-0.820*	0.178	-0.074	-0.573
Tissue, as a percentage of lamina thickness				
Adaxial epidermis	-0.282	0.394	-1.320	-2.774*
Abaxial epidermis	-0.685	-0.095	-1.566	-2.800*
Mesophyll	18.870	41.600*	2.547	-14.956
Palisade tissue	-19.613	-46.768*	-6.010	9.639
Spongy tissue	-21.299	-40.229*	-5.495	11.338
Palisade/spongy tissue ratio	-3.341*	2.005	-0.145	1.947
Cell cross-section area				
Adaxial epidermis	-0.324	-0.482	0.452	0.288
Abaxial epidermis	-0.280	0.223	-1.086*	0.025
Palisade tissue	-0.751	-1.011*	-0.351	-0.425
Spongy tissue	-0.198	-0.105	-0.611	0.261
Cumulative percentages of the vectors	47.32	64.26	76.49	88.56

negatively influence photosynthetic tissue and the process of photosynthesis. Thus, based on anatomical investigations, lucerne could be successfully grown with pea as a cover crop. From the anatomical perspective, these findings could contribute to developing a new, reliable and environmentally friendly method of the lucerne establishment.

Materials and methods

Experimental site

This research was a part of the trial conducted at the experimental field of the Institute of Field and Vegetable Crops at Rimski Šančevi, Serbia (45° 20' N and 19° 51' E, 84 m ASL). This site is characterized by a slightly carbonated chernozem soil of pH 7.1, as well as with the average annual rainfall of 624 mm and the average annual temperature of 11.2 °C (366 mm and 17.9 °C in growing season, April-September).

Treatments and experimental design

The trial was performed in rainfed conditions. The plot size was 6 m^2. Lucerne (cv. Mediana) was undersown crop with the field pea as the companion crop. Both undersown and cover crop were sown on March 29th, 2008. The design adopted was a two-factor trial with four randomized blocks, in addition to two control treatments. The first factor was field pea cultivar, whereby two cultivars, that differed in morphology of the leaves were chosen, based on the assumption that they could affect the undersown crop differently. The chosen cultivars were Jezero (afila type) and Javor (normal leaves with small leaflets). The second factor was a number of field pea plants, with 30, 60 and 90 plants per m^2. Lucerne was planted using a seeding rate of 15 kg ha^{-1}. The control I was pure stand of lucerne, as a standard mode of establishment, whilst the control II was lucerne sown with oats (*Avena sativa* L.), as a traditional method of lucerne establishment in the West Balkan Countries. Pea was sown first, at a depth of 4 cm, in rows 20 cm apart. Lucerne was subsequently sown between the pea rows, at 2 cm depth, reducing the distance between rows to 10 cm.

Measurements and experimental analysis

The samples were taken and the crop was harvested on the same day (June 10th, 2008) when the field pea cultivars reached the appropriate stage for forage production. Plants were cut to a stubble height of 7 cm. Ten lucerne plants from

each treatment were selected for anatomical studies. The middle leaflets of the trifoliate leaves from the middle part of the plants were separated and fixed in 50% ethanol. Cross-sections of ten leaves were made using Leica CM 1850 cryostat, at temperatures -18 °C to –20 °C, at cutting intervals of 25 μm. Light microscopy observations and measurements were performed using Image Analyzing System Motic 2000. Data were statistically processed and analysis of variance performed using STATISTICA for Windows version 8.0. Relative proportions of tissue thickness were calculated and expressed as a ratio to the full lamina thickness. The general structure of sample variability was established by Principal Component Analysis (PCA), based on correlation matrix. Multivariate Discriminant Function Analysis (MDA) was performed in order to assess the differentiation between the lucerne plants at the level of lamina anatomical characteristics.

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