Field quantification of foliar chlorophyll content in Pisum germplasm

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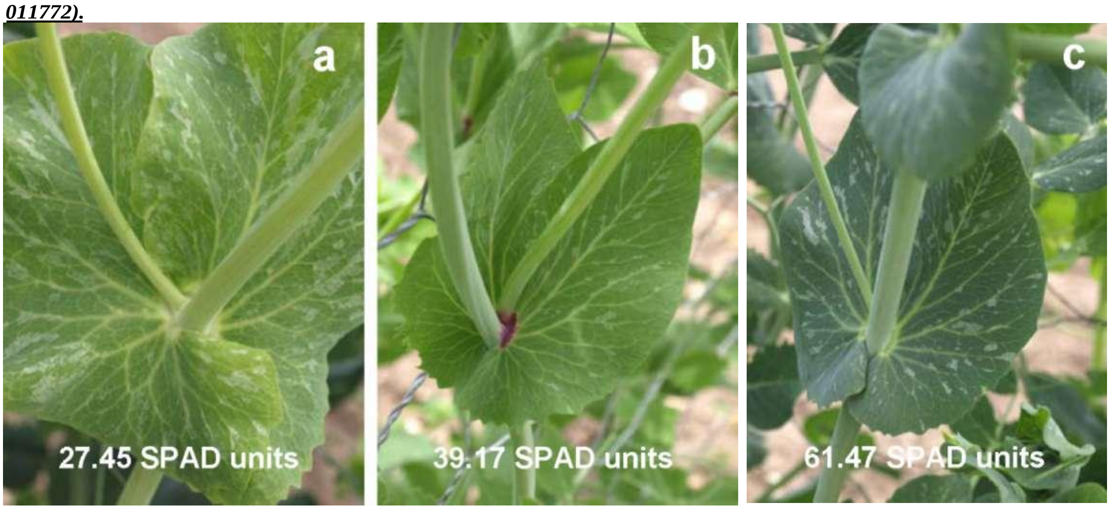
Variation in the chlorophyll content of the foliage of peas has been long documented. Traditional quantification of chlorophyll levels in leaves is by acetone extraction and spectrophotometer analysis (1) which takes time and requires laboratory equipment and facilities. In the classification of cultivated germplasm, the variation in the colour of foliage is graded based on morphological descriptor states. The UPOV guidelines for distinctness, uniformity and stability for Pisum (2) recognises three descriptor states, yellow green (J), green (2) and blue green (3). State *2* (green) is further broken down into light (3), medium (5) and dark (7). Three descriptor states are used in recording on the John Innes Pisum Collection namely, 1. *yellow green,* 2. *green,* 3. *dark green.* While these scales are clearly discernable by eye, a quick and reliable objective method of quantifying this variation could be useful in quantifying this character. The portable Minolta SPAD 502 chlorophyll meter determines the relative chlorophyll in leaf tissue by measuring absorbance at two wavelengths, namely in the regions of 400-500nm and 600-700nm which are characteristics of chlorophyll absorption peaks. Initially developed for monitoring the nitrogen status of wheat crops, the meter has subsequently been deployed on a range of monocot crop and woody species where good linear relationships between SPAD readings and leaf chlorophyll content were obtained (3). The method has also been used in crop nitrogen studies in pea (4, 5, 6) and in studies in chickpea (7, 8). This is the first deployment of the meter on pea germplasm in order to establish whether its utility could be extended to studies of pea germplasm and mutation stocks.

1. Survey of descriptor states for leaf colour

Readings were collected from a reference set of morphological variation in pea growing in the field against wire. A representative accession for each of the three descriptor states used when recording on the John Innes Collection were all assessed on the same day in early June using the fully expanded leaflets and stipules 3-4 nodes below the shoot apex. The reading showed a range of 32 SPAD units between the lowest (27 SPAD) and the highest (59 SPAD) with clear separation between each of the three colour classes (Fig. 1).

Figure 1. Variation in foliage colour for threeJI accessions growing in the field. a. yellow green (JI799), b. green (JI1889) and c. dark green 011772).



Readings were taken from the same accessions two weeks after the initial set at the end of flowering (end of June). The two sets of readings were very similar with the same levels and differentials between lines evident throughout (data not shown).

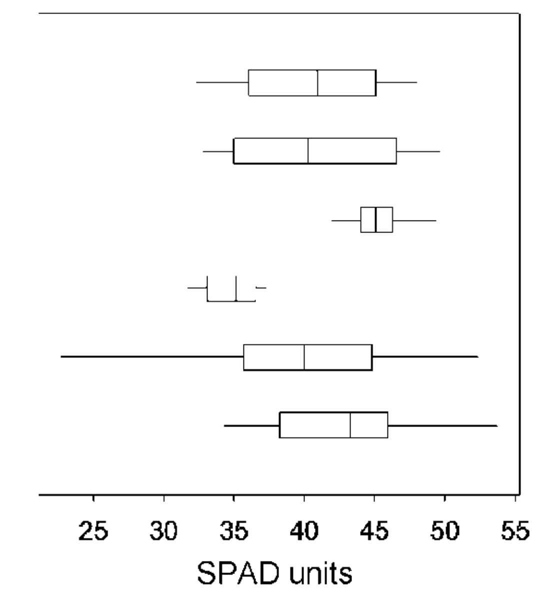
The area of leaf tissue that is clamped in the meter has a diameter of 13mm and an aperture for the light beam to pass through of 2mm x 3mm. This raised the question as to whether the variable degrees of grey flecking on foliage which is varies greatly between different accessions might possibly interfere with readings thus leading to high deviations across a leaf surface in the readings proved unfounded. The meter produced consistent readings between leaflets and stipules where there is frequently a differential degree of flecking. Flecking is caused by air spaces underlying the outer epidermis (9). The expression of flecking varies from totally absent which is encountered infrequently, through to a near continuous airspace as in the mutant argentum *(Arg)* characterised by the even silvery appearance of all foliage. Leaflets of the type line for *Arg* (JI 1397) gave SPAD readings for intact leaflets of 53, 46.4 and 48 which were in the middle to high range for peas. Reading across an individual leaflet where the outer epidermis was intact provided readings of 48, 44 and 47 SPAD compared to SPAD readings of 44, 45 and 46 across the area where it had been removed. The very slight reduction in the readings was not significant and converse to what might have been expected as the removal of the epidermis to expose the underlying mesophyll cells results in a significantly greener looking tissue.

2. Survey across the JI Pisum Test Array

The opportunity was taken to take readings from a test array of broad *Pisum* taxonomic diversity that was growing in the field at the same time (10). This consisted of 6 plants of 56 lines grown as clumps. Two measurements were taken on the 16/06 (all lines were in flower) from different plants of each accession which were then averaged. The range of SPAD values over the lines ranged from a minimum of 22.65 units (JI 102) to a maximum of 53.8 units (JI 399) with a mean of 41.46 units and a standard deviation of 6.39 units. The lines were then grouped on the basis of taxon or geographic region of origin (Fig. 2).

Figure 2. Descriptive statistics and boxplots of SPAD units for Pisum taxa and geographic groupings.

Taxon			
Pisum fulvum			1.
(n=4	Mean 40.58	St. Dev. 6.51)	
Pisum elatius			2.
(n-11	Mean 40.70	St. Dev. 6.0)	
Pisum abyssinicum			3.
(n-5	Mean 45.29	St. Dev. 7.10)	
Pisum sativum ssp. *transcaucasicum*			4.
(n-4	Mean 34.81	St. Dev. 2.66)	
Pisum sativum (Asiatic highlands)			5.
(n-10	Mean 39.75	St. Dev. 8.98)	
Pisum sativum var. *sativum*			6.
(n-22	Mean 43.13	St. Dev. 5.50)	



This highlighted the relative high readings obtained for the four accessions of *Pisum abyssinicum* which as a group had a mean of 45.29 units ± st. dev.7.10. All 5 accessions of this taxon share an absence of grey flecking on the foliage. To the naked eye, this material appeared lighter than the SPAD readings indicated suggesting this taxon is different in some way for the epidermal cell layer or in the epicuticular wax. The 4 accessions of *Pisum sativum* spp. *transcaucasicum* were noticeably paler green as a group which was reflected in the readings (34.81 units ± st. dev. 2.66). The sativum lines from the Asiatic highlands were separated out as they clearly form a distinct ecogeographic group which included 4 accessions of the 'Afghanistan type'. This distinct form of cultivated pea is noted for variation in their ability to nodulate with European strains of Rhizobium Lines resistant to nodulation are easily noted as becoming precociously yellow during the pod filling stage due to the total reliance on available soil nitrogen (ll, 12). This is regularly observed in such material in the low fertility sandy loam of the experimental plots at the Jic. Four 'Afghan type' accessions scored by Young and Matthews (12) were present in the test array and one line in particular (JI 102) was noticeably yellow. The data for these four accessions is presented in Table l and shows the two accessions resistant to nodulation provided lower SPAD values.

Table 1. Nodulation response to European rhizobium and SPAD units for 4 accessions of 'Afghan type' peas.

JI Acc. No.	Nodulation by European Rhizobium	SPAD unit readings
85	Partial	41.3, 43.1
109	Partial	39.8, 35.9
95	Resistant	33.5, 27.4
102	Resistant	20.2, 25.1

The number of data points was too low to perform significance tests but the readings obtained for the two groups were non-overlapping.

3. Survey of genetic stocks of leaf chlorophyll mutants

In the autumn a glasshouse survey was undertaken on a range of type lines and mutants for genes associated with foliage chlorophyll content. This included four lines that had been measured in the field earlier in the summer (section l). For this survey, three readings were taken from separate leaflets (2nd true leaf) from three separate plants. The plants were expanding their leaflets at the fifth vegetative node with the exception of JI 35 (yellow green) where the measurements were taken from the youngest expanded leaflets at the top of the plant (node 4) where the phenotype is expressed (13, 14).

No significant differences were found between the readings obtained in the field or glasshouse showing the SPAD reading to be consistent for these lines across these independent sowings and environments (Table 2).

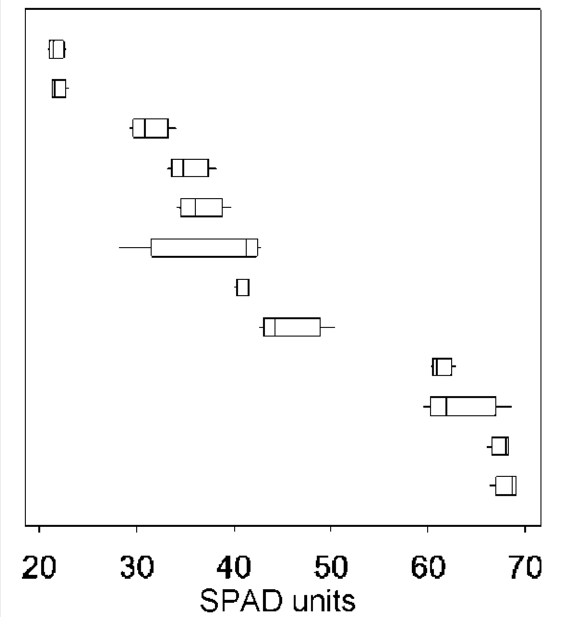
Table 2. Paired t-tests on SPAD unit values for four JI accessions grown in the field and glasshouse.

JI Acc. No.	Mean SPAD Units (Field grown)	Mean SPAD Units (Glasshouse grown)	P and Significance
799	27.45	31.4	0.233 (N.S).
1889	39.17	36.63	0.035 (N.S.)
1772	61.47	61.33	0.978 (N.S.)
1397	49.13	45.8	0.015 (N.S.)

In this survey of 12 lines (Fig. 3), a threefold range of values obtained from the palest line (JI 2414) to the darkest (JI 1405). JI 2414 is a genetically uncharacterised stable chlorophyll b mutant where the whole plant is a uniform bright light green (15). This was lower than the type line for *o* (JI 799) which is a pale yellow green and gave an mean value of 31.4 SPAD units. The highest SPAD readings obtained were for the *cov* type line (JI 2729) and JI 1405 which is equally dark green in appearance came with mean SPAD values of 67.46 and 61.8 respectively.

Figure 3. SPAD units for ranked JI accessions grown in the glasshouse. Gene symbols in parentheses and provisional pending allelism tests.

Ranked order	JI Acc. No.	SDAP units (mean)	Genotype
1	2414	21.73	*(chl)*
2	35	21.96	*ygl*
3	799	31.4	*o*
4	2687	35.43	*pa*
5	1889	36.63	
6	322	37.46	*pa, Vim*
7	825	41	*ccr*
8	1397	45.8	*Arg*
9	1772	61.33	
10	61	63.36	*Pa,vim*
11	2729	67.46	*cov*
12	1405	68.1	*(cov)*



While this is a small survey of mutant stocks from the total number of chlorophyll mutants isolated in pea, they serve to demonstrate the range of greens of foliage observed in chlorophyll contents. The studies outlined in this paper demonstrate the suitability of the SPAD-520 chlorophyll meter for working with Pisum over a wide range of intensity of green coloured foliage. The meter is highly portable and proved easy to calibrate and quick and reliable to use. While differences at the extremes of the range and the mid point are easy to score by eye, variation within material in the mid-range is much harder. It is in this range or having an objective measure of chlorophyll across sites and/or years where this device might prove particularly useful.

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Potential of the intercrops of normal-leafed and semi-leafless pea cultivars for forage production

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Pea *(Pisum sativum* L.) is one of the most ancient food and feed crops in the Balkan Peninsula. Its traditional use in animal husbandry on the territory of modern Serbia has been in the form of forage, either fresh or dry, as well as in mixtures with small grains or as a pure stand (1). The first Serbian forage pea cultivars were autumn-sown, with excellent winter-hardiness and delayed flowering resulting in high quality and stable forage yields (2). The semi-leafless types of pea entered the Serbian market and breeding programs during the last decade of the last century and are exclusively associated with dry grain production. Recently, it was demonstrated that it is possible to develop autumn-sown semi-leafless dry pea cultivars for the conditions of Serbia (3), and that the semi-leafless cultivars may provide high quality forage yields with enhanced seed production due to their prominent lodging tolerance (4).

Numerous contemporary systems involving monocultures require chemical fertilizers and pesticides, and result in decreased soil and water quality and reduced biodiversity. On the other hand, intercropping systems with carefully and appropriately selected species where legumes serve a prominent role provide many advantages in contrasting environments (5). Along with the traditional intercropping of legumes with small grains in many European regions (6, 7), attempts have been made recently to examine intercrops of perennial and annual legumes, such as field pea in establishing red clover (8), as well as mutual intercropping of annual legumes for forage production (9). The goal of this research was to assess the potential of the mutual intercrops of pea cultivars with different leaf types for forage production.

Materials and Methods

A small-plot trial was established during the 2008-2009 and 2009-2010 growing seasons at Rimski Sancevi, near Novi Sad (45°20' N, 19°51' E and 84 masl) on a slightly carbonated chernozem soil (Table 1). Compared to the long term average (1964-2010), the growing season of 2008/09 was much warmer and much drier, while the growing season of 2009/10 was slightly warmer and significantly wetter (Table 2).

Table 1. Basic chemical properties of a chernozem soil at Rimski Šančevi.

Depth (cm)	pH KCl	pH H_2O	$CaCO_3$ (%)	Humus (%)	N total (%)	Al-P_2O_5 (mg 100g^{-1})	Al-K_2O (mg 100g^{-1})
0-30	7.41	7.90	5.61	2.97	0.196	17.99	20.00

Table 2. Average monthly temperatures (°C) and monthly precipitation (mm) for the growing period of autumn-sown cool season annual forage legumes in 2008/2009 and 2009/2010.

Month / Year	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Mean
Average monthly temperature (°C)									
2008/2009	14	9	4	2	6	7	15	18	9.4
2009/2010	12	9	3	0	2	7	13	17	7.9
Long term	12	6	2	-1	2	6	11	17	6.9
Monthly precipitation sum (mm)									Total
2008/2009	17	58	45	23	7	36	2	48	236
2009/2010	83	64	96	73	65	38	71	95	585
Long term	43	50	48	37	32	38	47	59	354

The trial included four dry pea cultivars in total, namely the autumn-sown semi-leafless cultivar 'Dove' (France), the autumn-sown normal-leafed cultivar 'Frijaune' (France), the spring-sown semi-leafless cultivar 'Jezero' (Serbia) and the spring-sown normal-leafed cultivar 'Javor' (Serbia). These four cultivars were grown in pure stand and intercropped with the complementary genotype within the same crop type such that one had good standing ability (supporting crop, Dove and Jezero) while the other was lodging-susceptible (supported crop, Frijaune and Javor). There were six treatments in total: four pure stands and two intercropped treatments, i.e. Dove with Frijaune and Jezero with Javor.

The trial was set up as a randomized complete block design, with four replicates, and plot size of 10 m^2 and a row spacing of 20 cm. The pure stands were sown with 120 viable seeds m^{-2}. The intercropped treatments were composed of 50% of each variety. The autumn treatments were sown on October 11, 2008, and October 15, 2009, while the spring treatments were sown on March 14, 2009, and March 22, 2010.

Pure stands were cut at full bloom, while the intercrops were cut when the first of the two components reached full bloom. The varieties in the combined treatments flowered at similar times. The autumn-sown treatments were cut on April 17, 2009, and April 25, 2010, while the spring-sown treatments were cut on May 7, 2009, and May 17, 2010. Green forage yield (t ha^{-1}) and forage dry matter yield (t ha^{-1}) were monitored. The Land Equivalent Ratio (LER) was calculated as (10):

$$LER = Sdic / Sdps + Sgic / Sgps$$

, where Sd_{IC} is the supported component forage yield in the intercrop, Sd_{PS} is the supported component forage yield in its pure stand, Sg_{IC} is the supporting component forage yield in the intercrop and Sg_{PS} is the supporting component forage yield in its pure stand. The values for both green forage yield (LER_{GFY}) and forage dry matter yield (LER_{FDMY}) were calculated separately.

The analysis of variance was performed using Statistica 8.0 software, with a Fisher's LSD test used at P = 0.05.

Results and Discussion

There were statistically significant differences at the p = 0.05 level between green forage yield among treatments (Table 3). On average, green forage yields were higher in the 2009/10 growing season compared to the 2008/09 growing season. Pure stand green forage yield varied from 28.5 t ha^{-1} for the normal-leafed spring-sown Javor in 2008/09 to 34.0 t ha^{-1} for the spring-sown semi-leafless Jezero also in 2008/09. In the 2008/09 growing season, the autumn-sown and the spring-sown intercrops produced similar total green forage yield (33.9 t ha^{-1} and 33.8 t ha^{-1}), while, in the 2009/10 growing season, the autumn-sown intercrops produced higher yields (34.5 t ha^{-1}) than the spring-sown intercrops (33.9 t ha^{-1}). The supported component of the autumn-sown intercrops benefitted much more than the supporting crop in both years, while in the spring-sown intercrops the two component varieties were more balanced in their contribution to the total green forage yield. These results suggest that semi-leafless pea cultivars may have great potential for forage production (11).

Both autumn-sown and spring-sown intercrops had two-year LER_{GFY} average values greater than 1 (1.09 and 1.11) suggesting that intercropping the two varieties was economically justified for green forage production.

Table 3. Green forage yield (t ha^{-1}) and LER_{GFY} in mutual intercrops of pea cultivars with different leaf types at Rimski Šančevi during the 2008/09 and 2009/10 growing seasons.

Year	Season	Treatment	Green forage yield of supported component	Green forage yield of supporting component	Total green forage yield	LER_{GFY}
2008/ 2009	Winter	Dove, pure stand	31.6	0.0	31.6	1.00
		Frijaune, pure stand	0.0	30.4	30.4	1.00
		Dove +Frijaune	22.2	11.7	33.9	1.10
	Spring	Jezero, pure stand	34.0	0.0	34.0	1.00
		Javor, pure stand	0.0	28.5	28.5	1.00
		Jezero + Javor	18.3	15.5	33.8	1.10
2009/ 2010	Winter	Dove, pure stand	33.2	0.0	33.2	1.00
		Frijaune, pure stand	0.0	31.2	31.2	1.00
		Dove +Frijaune	24.1	10.4	34.5	1.09
	Spring	Jezero, pure stand	28.6	0.0	28.6	1.00
		Javor, pure stand	0.0	32.0	32.0	1.00
		Jezero + Javor	14.4	19.5	33.9	1.13
Average 2008/ 2010	Winter	Dove, pure stand	32.4	0.0	32.4	1.00
		Frijaune, pure stand	0.0	30.8	30.8	1.00
		Dove +Frijaune	23.2	11.0	34.2	1.09
	Spring	Jezero, pure stand	31.3	0.0	31.3	1.00
		Javor, pure stand	0.0	30.3	30.3	1.00
		Jezero + Javor	16.4	17.5	33.8	1.11
$P < 0.05$			3.7			0.08

Average forage dry matter yields in pure stands were statistically significant (Table 4). Yield ranged from 5.5 t ha^{-1} in the spring-sown semi-leafless Jezero in 2008/09 and 8.0 t ha^{-1} in the autumn-sown, normal-leafed Frijaune in 2009/10. The trend of forage dry matter yield was not the same as green forage yield due to different forage dry matter proportion in individual cultivars and individual years.

In 2008/09, forage dry matter yield in the autumn-sown intercrop (7.7 t ha^{-1}) was significantly greater compared to the forage dry matter yield in the spring-sown intercrop (6.4 t ha^{-1}). In 2009/10, the autumn-sown intercrop was significantly more productive (8.4 t ha^{-1}) than the spring-sown intercrop (6.5 t ha^{-1}). Similar to the case of green forage yield, there was a balance between the two components in the spring-sown intercrop, while the supported component had much greater contribution in the autumn-sown intercrop.

The LER_{FDMY} values suggest that both autumn-sown and spring-sown intercrops were economically justified, although the former was significantly more productive (1.13) than the latter (1.03).

Conclusions

The obtained results give a solid basis for further research on intercropping legume varieties with contrasting leaf types for forage production. One of the advantages the mutual dry pea intercropping may have in comparison to the traditional annual forage legume cultivation is a prominent earliness, especially in the autumn-sown treatments, allowing the possibility of sowing a succeeding crop in a regular sowing term. Future research in the mutual pea and other annual legume intercropping must include evaluation of additional above and below ground characteristics.

Table 4. Forage dry matter yield (t ha^{-1}) and LER_{FDMY} for mutual intercrops of pea cultivars with different leaf types at Rimski Šančevi during the 2008/09 and 2009/10 growing seasons.

Year	Season	Treatment	Forage dry matter yield of supported component	Forage dry matter yield of supporting component	Total forage dry matter yield	LER_{FDMY}
2008/ 2009	Winter	Dove, pure stand	6.7	0.0	6.7	1.00
		Frijaune, pure stand	0.0	7.6	7.6	1.00
		Dove +Frijaune	4.9	2.8	7.7	1.10
	Spring	Jezero, pure stand	6.3	0.0	6.3	1.00
		Javor, pure stand	0.0	5.5	5.5	1.00
		Jezero + Javor	3.0	3.4	6.4	1.09
2009/ 2010	Winter	Dove, pure stand	6.9	0.0	6.9	1.00
		Frijaune, pure stand	0.0	8.0	8.0	1.00
		Dove +Frijaune	5.3	3.1	8.4	1.16
	Spring	Jezero, pure stand	7.1	0.0	7.1	1.00
		Javor, pure stand	0.0	6.5	6.5	1.00
		Jezero + Javor	2.7	3.8	6.5	0.96
Average 2008/ 2010	Winter	Dove, pure stand	6.8	0.0	6.8	1.00
		Frijaune, pure stand	0.0	7.8	7.8	1.00
		Dove +Frijaune	5.1	3.0	8.1	1.13
	Spring	Jezero, pure stand	6.3	0.0	6.3	1.00
		Javor, pure stand	0.0	6.4	6.4	1.00
		Jezero + Javor	2.9	3.6	6.5	1.03
$P < 0.05$				0.8		0.08

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