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Pisum Genetics Association Notes

The Pisum Genetics Association (PGA) is a non-profit, unincorporated organization established to foster genetic study of the pea, to facilitate the exchange of information and to ensure preservation of valuable genetic stocks. The journal, **Pisum Genetics**, is the principal means of meeting these aims. Published annually in one issue, Pisum Genetics contains reports of research findings and other information of interest to researchers studying the genus *Pisum*.

Membership and Dues

Membership in the PGA is open to all interested persons and organizations. Dues are US \$15.00 per year. These dues are used to help defray the costs of publishing and mailing Pisum Genetics. Members are requested to send checks or bank drafts in US currency only. Such efforts avoid bank charges, which can exceed \$10.00 per check in some currencies. Please make checks payable to Pisum Genetics Association and send to Kevin McPhee, NDSU Dept 7670, P.O. Box 6050, 370G Loftsgard, Fargo, ND, USA, kevin.mcphee@ndsu.edu. Pisum Genetics will be sent upon receipt of dues.

All members of the PGA are welcome to submit manuscripts on topics appropriate for Pisum Genetics. The deadline for such submittals is usually September 1 of the year of publication, allowing for sufficient time for review and revision of manuscripts. The journal provides opportunities for both refereed and non-refereed manuscripts, publication is within 3 to 6 months of submittal, and there are no page charges. Pisum Genetics is indexed in CAB International. As has been the case for the last decade, a web-based version is available shortly after publication of the hard copy (<http://hermes.bionet.nsc.ru/pg/>).

Submission of Manuscripts

When submitting an electronic copy of a manuscript, please use Microsoft Word when possible. Other formats can be handled, but these will usually be converted to Word for processing. It is also helpful if authors avoid the use of extra formatting in their submissions (headers, sections, automatic indentation) as these generally have to be eliminated when preparing the manuscript for final printing. Tables created in Word using the table function are most likely to survive the formatting process intact. We have severe limitations on support staff, and the removal of interfering formatting can occasionally be quite time-consuming. Graphics reproduce best at 300 dpi.

Format of Manuscripts

Manuscripts are generally separated into two types, research papers and brief communications. Manuscripts submitted as research papers will be subjected to peer review. Research papers should be written concisely, with a short introduction presenting the purpose for the study, a materials and methods section with essential details and references to techniques, a results section and discussion section interpreting the results and integrating findings with those of other workers. Section headings are not obligatory. The length of manuscripts is flexible. Manuscripts under five journal pages are encouraged. Longer papers will be considered on their merits and space availability in the volume. All research papers must be treated similarly to submissions to other refereed journals in that they should contain original work not currently submitted to any other journal.

**Brief
Communications**

Brief communications are to encourage a rapid sharing of new results that may not meet the standards of a research paper (i.e. allelism tests not completed, map locations only approximate). Brief communications can also be used to describe the availability of new germplasm. Brief communications will not be subjected to peer review although the editor reserves the right to screen papers for appropriateness and to review submissions for clarity and brevity. Such submissions will be limited to approximately one page and should be narrowly focused. Otherwise, the format of brief communications (tables, references, etc.) should correspond to that of a research paper. Publication of a brief communication in Pisum Genetics does not preclude publication of a full paper on the same subject in a later issue or in another journal.

Member News

Dr. Serge Rozov continues to serve as the webmaster for the PGA website (<http://hermes.bionet.nsc.ru/pg/>) and has extended the coverage of previous issues available on the site back to volume 19 (1987). Electronic versions of volumes 17 and 18 are expected to be available on this site shortly. Hard copies of volumes 10 through 20, plus a selected number of earlier volumes can be obtained by contacting the editorial office.

Financial Report

The PGA continues to be fiscally healthy. Income from dues and subscriptions has been adequate to cover expenditures. We have adequate funds in savings/certificate of deposit accounts that can cover most PGA expenses for several years. The financial accounts were transferred to Fargo, North Dakota, in late 2008 and are expected to be maintained there for the foreseeable future. The financial report is based on the transactions for 2010 through September 30, including dues and publication costs for Volume 41. Expenditures for publication and distribution of Volume 42 are not included in this report.

Financial Report

Account balances from preceding year			
	Checking (Fargo)	1577.85	
	Savings/Certificate of Deposit (Fargo)	6496.49	
	Total assets		8074.34
 Income			
	Subscriptions (Fargo)	465.00	
	Bank interest (Fargo)	93.93	
	Total income		558.93
 Expenditures			
	Printing costs (volume 41)	870.26	
	Mailing expenses (volume 41)	514.18	
	Total expenditures		(1384.44)
 Account balances			
	Savings/Certificate of Deposit (Fargo)	6590.42	
	Checking (Fargo)	658.41	
 Net balance for Pisum Genetics Association			7248.83

K.E. McPhee for the Coordinating Committee:

M. Ambrose	K.E. McPhee	S.M. Rozov
T.H.N. Ellis	I.C. Murfet	W.K. Świącicki
O.E. Kosterin	J.B. Reid	N.F. Weeden

Identification of tolerance to *Fusarium* root rot in wild pea germplasm with high levels of partial resistance

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Fusarium root rot, caused by *Fusarium solani* (Mart.) Sacc. f. sp. *pisi*, (*Fsp*) is a serious seed and root rot disease affecting pea growing areas throughout the world (1, 2, 3). The disease may damage peas produced in both dry land and irrigated fields, and has been reported to reduce yield between 30 to 57% in eastern Washington, U.S.A. and Canada (3, 4, 5). The pathogen infects the cotyledon region and spreads downward to the roots and upward to the crown. Early disease symptoms include reddish brown to black lesions on the roots that often expand and coalesce as the growing season progresses towards harvest. Mature plants may be severely stunted or die due to infection.

The commercial pea cultivars currently available have not been specifically bred for resistance to *Fsp*. However, several pea germplasm lines with partial resistance have been released (6, 7, 8, 9, 10), and resistant cultivars are being developed from these sources. In addition, new sources of partial resistance to *Fsp* have been identified in *Pisum sativum* ssp. *elatius* var. *pumilo* (11) and in 44 accessions from the *Pisum* Core Collection in 2003 (12). The partial resistance of these 44 accessions was characterized solely on root disease severity ratings from 0 to 5 (0 = no infection). Quantitative measurements of these 44 accessions comparing plant germination rates, plant height, fresh and dry foliage weight, and root dry weight between inoculated and non-inoculated plants of the same accessions were never assessed. Such comparisons would provide additional valuable information to characterize pea accessions that are not only highly resistant to root rot, but also tolerant to *Fsp*, with tolerance being defined as the ability of the infected plants to maintain normal growth (growth that is not significantly different from that of healthy plants when assessed under the same conditions) although infected by the pathogen.

The hypothesis of this research was that tolerant lines existed among the 44 *Pisum sativum* accessions that had previously been determined to have partial resistance to *Fusarium solani* f. sp. *pisi* (12). Identification of these lines would improve the selection of wild pea germplasm with both high partial resistance and tolerance to *Fsp* that could be used by pea breeders to select the most promising germplasm to be used to incorporate new genes into breeding programs to improve resistance to *Fsp*.

Materials and Methods

Pea seed of wild pea germplasm of forty-four accessions were obtained from the *Pisum* Core Collection (United States Department of Agriculture, Western Regional Plant Introduction Station, Pullman, WA, USA). These accessions had previously been determined to have some level of disease resistance to *Fsp* based on a 2003 survey of the *Pisum* Core Collection (12). The accessions were comprised of *Pisum sativum* (41 accessions), *Pisum sativum* subsp. *sativum* (2 accessions) and *Pisum sativum* var. *arvense* (1 accession). The accessions represented germplasm originating from fourteen countries (Table 1 provides information on selective pea genotypes from among the 44 accessions that demonstrated partial resistance and tolerance based on the present research).

The *Fsp* isolates F54, FS-01-B1, and F215, from our culture collection were used for inoculum in this study. These isolates originated from infected pea plants from three separate locations within Washington State, USA. Macroconidia of these isolates were mass produced following the below mentioned inoculum procedure and stored at 5°C in 10 ml test tubes containing 10 g of a sterile 1:1:1 (vol/vol/vol) soil:peat moss:perlite mixture (13). Inoculum was prepared for each isolate separately by transferring infested soil grains from the 10 ml test tubes onto peptone-pentachloronitrobenzene agar (PCNB) (14) in 9 mm Petri

Table 1. PI accession number, origin and flower/seed color of selective wild pea germplasm of *Pisum sativum* maintained in the Pisum Core Collection as part of the Western Regional Plant Introduction Station located in Pullman, WA, U.S.A., in which the accessions were screened for resistance to *Fusarium solani* f. sp. *pisi*.

PI accession ^a	Origin	Flower/seed color ^b
PI125839	Afghanistan	p/p
PI125840	Afghanistan	p/p
PI175226	India	p/m
PI184128	Yugoslavia	p/p
PI198735	Afghanistan	p/p
PI220174	Afghanistan	p/p
PI220189	Afghanistan	p/p
PI222071	Afghanistan	p/p
PI222117	Afghanistan	p/p
PI223526	Afghanistan	p/p
PI223527	Afghanistan	p/p
PI226561	Ethiopia	p/p
PI227258	Iran	p/p
PI271119	China	p/p
PI166159	Nepal	p/p
PI257593	Ethiopia	p/p
Bolero	NA	w/np
DSP	NA	w/np

^a Two moderately resistant PI accessions (PI257593 and PI166159) and two susceptible pea cultivars (Bolero and Dark Skin Perfection) to *Fusarium solani* f. sp. *pisi* were used as standards in each test. Information on accessions was obtained from GRIN - Germplasm Resource Information Network, United States Department of Agriculture.

^b m = mixed flower color, np = non-pigmented (green/white) seed coat, p = pigmented, w = white flower.

ten wet seeds of each inoculated and non-inoculated genotype were planted in separate plastic trays (52.5 by 26.25 by 6.25 cm) filled with propagation grade-course perlite (Supreme Perlite Company, Portland, OR). Each tray contained three inoculated or three non-inoculated pea genotypes, each planted in the trays lengthwise in single rows with 10 plants per row. There were three replications of each genotype per test that were inoculated and non-inoculated, and each test was repeated one or two times for a maximum of three separate screening tests per genotype. Due to greenhouse space constraints, the forty-four accessions screened for *Fsp* resistance were divided into four tests labeled 1 through 4, with eleven different PI accessions screened per test. Multiple screenings of the same genotypes conducted at different times were identified as Test 1a, Test 1b and Test 1c. In addition to the eleven accessions screened per test, each test also contained two *Fsp*-susceptible pea cultivars (Dark Skin Perfection and Bolero) and two *Fsp*-moderately-resistant PI accessions (PI257593 and PI166159) as controls, except in Test 1a and 1b, where only Dark Skin Perfection was used as a control.

The plants were grown in a greenhouse under natural sunlight, and supplemental lighting with 1000-watt metal halide lights used as needed to maintain a photoperiod of approximately 14 hours. The greenhouse temperatures for each test ranged from 15 to 28°C. The plants were watered uniformly as needed with approximately 500 ml of water per tray and fertilized uniformly with 500 ml of Miracle-Gro (24-8-16, N-P-K) (Marysville, OH) at a concentration of 4.93 ml Miracle-Gro per liter of water at 9, 12, 15, 18 and 21 days after planting.

The pea plants were harvested 25 days after planting, which allowed sufficient time for root rot symptoms to develop, and disease resistance was characterized based on the following comparisons between inoculated and non-inoculated plants of each accession: 1) percent germination of seed, 2) root disease

plates. The plates were incubated for eight days at 23°C under continuous florescent cool white 34-watt lights. Following incubation, three plugs (4 mm in diameter) containing mycelia were transferred from the agar plates into 120 ml flasks containing Kerr's Medium (15). The flasks were placed on a shaker for seven days at 23°C under continuous florescent cool white light. Cultures of each isolate were then strained through one layer of cheesecloth and centrifuged at 2500 rpm for 5 minutes. The supernatant was poured off, and the spores were re-suspended using sterilized distilled water. The conidia concentrations for each isolate were determined using a hemacytometer, and the suspensions were adjusted to 1×10^6 conidia per/ml of water. Prior to inoculation, equal volumes of the isolates were combined into a single suspension and the seeds were immediately inoculated.

Fifty seeds of each pea genotype were placed in each of two 100 ml beakers to which 60 ml of a conidial suspension of *Fsp* were added to one beaker and 60 ml of sterilized distilled water were added to the other. The seed were soaked for 17 h at 25°C. The seed of each genotype soaked in sterilized distilled water were used as non-inoculated controls. Following the soaking period,

severity (RDS), 3) foliage fresh weight, 4) foliage dry weight and 5) root dry weight. RDS values were assessed using a 0 to 5 scale: 0 = no infection; 1 = 1-10% infected root area (IRA); 2 = 11-25% IRA; 3 = 26-50% IRA; 4 = 51-80% IRA; 5 = 81-100% IRA (16). The foliage and the root dry weights were obtained by placing root and foliage samples in individual paper bags and placing the samples in a dryer at 45°C for 72 hours before weighing.

The greenhouse screening technique used to identify *Fsp* resistance in these tests is a refined method developed by Dr. John Kraft based on a previously published technique (16). This screening method has been used successfully to identify *Fsp*-resistant pea lines screened under greenhouse conditions, that are also *Fsp*-resistant when screened in the field (7, 8).

The general linear model (PROC GLM) in SAS (SAS Institute Inc., Cary, NC) was used to analyze the disease and plant parameters that were measured. Mean pair-wise comparisons among accessions were made using Fisher's Least Significant Difference Test ($P \leq 0.05$). A PROC RANK procedure in SAS was also used to rank accessions from 1 to 11 according to their RDS rating within each trial of each test. A rank of "1" was assigned to the accession with the lowest disease rating and an "11" to the accession with the highest rating.

Results

In tests 1, 2, 3 and 4 and their repeated screenings (a to c), the mean percent germination was not significantly different ($P > 0.05$) between inoculated and non-inoculated plants of the same accession in two or more screenings tests. However, the mean percent germination of the inoculated seed of both susceptible controls, Dark Skin Perfection and Bolero, was significantly less ($P < 0.05$) than the non-inoculated seed of these cultivars in 10 of 10 and 8 of 8 screenings, respectively, in which they acted as susceptible controls in tests 1 to 4 for all screenings (data not shown due to lack of significant difference in two or more test among the accessions).

The roots of all non-inoculated control plants for all the screening tests were free of root rot and were rated as zeros according to the RDS scale. In Tests 1a, 1b and 1c, the mean RDS of accession PI125839 was numerically less than all other inoculated accessions and was significantly less ($P < 0.05$) than the severity of nine or more accessions in trials 1a and 1b (Table 2). In Tests 2a, 2b and 2c, accessions PI184128 and PI198735 consistently demonstrated significantly lower ($P < 0.05$) mean RDSs than six other inoculated accessions screened in two or more of these tests (Table 2). In Tests 3a and 3b, accessions PI220174, PI220189, PI222071 and PI222117 consistently demonstrated the lowest mean RDSs in both tests that were either significantly ($P < 0.05$) or numerically less than five or more other inoculated accessions (Table 2). In Test 4a and 4b, the mean RDS of accession PI271119 was either numerically or significantly less ($P < 0.05$) than 9 or more of the inoculated accessions screened for both tests (Table 2).

The mean plant height, foliage fresh weight, foliage dry weight and root dry weight of the inoculated and non-inoculated plants were not significantly different ($P > 0.05$) from each other in two or more screenings for only eight (PI125839, PI125840, PI175226, PI220174, PI223526, PI223527, PI226561 and PI227258) of the 44 pea accessions including the controls (Table 3, only shows genotypes demonstrating no significant differences in growth parameters assessed between inoculated and non-inoculated genotypes).

Discussion

Previous research identified the 44 *Pisum* accessions assessed in the present study as having some partial resistance to *Fsp* based on low RDS ratings (12). This resistance was identified based on a single test, where two replications of 10 seed were used to screen each accession (12). Therefore, in the present study, more highly replicated screening tests were used to further characterize consistent resistance to *Fsp* root rot among these 44 wild *Pisum sativum* accessions. The present study identified accessions PI125839,

Table 2. PI accession number, screening test and root disease severity of selective wild pea germplasm from the Pisum Core Collection in Pullman, WA, USA demonstrating resistance to *Fusarium solani* f. sp. *pisi* when seed of forty-four accessions were inoculated and assessed 25 days after planting for root disease severity in repeated tests (Only the most consistently resistant PI accessions in the trials of each test are represented in the table).

PI accession # ^a	Screening test ^b	Root disease severity ^d		
		Replicate a ^c	Replicate b	Replicate c
PII25839	1	0.92 (1) ⁹	1.13 (1) ¹⁰	1.42 (1) ³
PII66159	1	...	1.64 ^{**e}	2.43 ^{**}
PI257593	1	...	2.32 ^{**}	2.47 ^{**}
Bolero	1	...	3.05 ^{**}	3.34 ^{**}
DSP	1	NG	4.50 ^{**}	3.00 ^{**}
RDS-Range	1	1.18-2.04	1.38-2.32	1.44-2.49
PII84128	2	1.58 (1) ⁶	1.57 (1) ⁶	2.32 (7) ⁴
PII98735	2	1.82 (4) ⁶	1.67 (2) ⁶	1.93 (2) ⁶
PII66159	2	...	2.27 ^{**}	2.32 ^{*i}
PI257593	2	...	2.93 ^{**}	2.29 [*]
Bolero	2	3.88 a	3.88 ^{**}	3.5 ^{**}
DSP	2	2.4 b	NG ^{**}	2.67 ^{**}
RDS-Range	2	1.59-3.05	1.67-3.91	1.74-3.69
PI220174	3	1.47 (5) ³	1.42 (1) ⁴	...
PI220189	3	1.36 (1) ⁴	1.53 (4) ³	...
PI222071	3	1.37 (2) ⁴	1.47 (3) ³	...
PI222117	3	1.38 (3) ⁴	1.46 (2) ³	...
PII66159	3	1.76 ^{**}	2.01 ^{**}	...
PI257593	3	1.73 ^{**}	2.24 ^{**}	...
Bolero	3	3.56 ^{**}	3.90 ^{**}	...
DSP	3	3.60 ^{**}	3.50 ^{**}	...
RDS-Range	3	1.42-1.97	1.65-2.53	...
PI271119	4	1.36 (1) ⁴	1.48 (2) ⁴	...
PII66159	4	1.69 [*]	1.71 [*]	...
PI257593	4	2.78 ^{**}	2.11 ^{**}	...
Bolero	4	4 ^{**}	3.75 ^{**}	...
DSP	4	4.17 ^{**}	NG ^{**}	...
RDS-Range	4	1.50-2.68	1.38-2.31	...

^a Italized names and PI accessions represent two moderately resistant (PI257593 and PII66159) and two susceptible (Bolero and Dark Skin Perfection) controls to *Fusarium solani* f. sp. *pisi*. DSP and Bolero are commercial pea cultivars. RDS-Range - provides the range of the root disease severity values of the other PI accessions screened within a given screening test, not including the most resistant accessions already provided in the table. DSP - Dark Skin Perfection.

^b Forty-four accessions were divided into four screening tests (1 to 4) and 11 accessions were screened per test. Tests were not screened at the same time but on different dates spaced at two-week intervals. Seed of inoculated plants and non-inoculated plants of each accession were treated with 1×10^6 macroconidia of *F. solani* f. sp. *pisi* and sterile distilled water, respectively. Roots of non-inoculated plants were free of infection.

^c Example = 0.92 (1)⁹ 0.92 = mean root disease severity value of the accession within a given trial. (1) = resistant rank among the 11 accessions screened per test based on mean root disease severity, with a rank of "1" being the most resistant accession and "11" being the least resistant accession to *Fusarium* root rot according to a PROC RANK procedure in SAS. ⁹ = Signifies that the mean root disease severity value of the accession was significantly less than ($P < 0.05$) the root disease severity value of 9 of the 11 accessions screened within a test according to a Fisher's LSD test. "..." = not included in the trial. NG = inoculated seed did not germinate due to infection by *Fusarium solani* f. sp. *pisi* based on comparisons with the non-inoculated water controls.

^d Root disease severity was based on a 0 to 5 scale, "0" = no infection, 1 = 1-10% infected root area (IRA); 2 = 11-25% IRA; 3 = 26-50% IRA; 4 = 51-80% IRA; 5 = 81-100% IRA. Roots of non-inoculated controls of each accession were clean in all the tests and rated as zeros for their root disease severity.

^e *** = the mean root disease severity value of the control (PII66159 in this case) is significantly greater ($P < 0.05$) than the *Fsp*-resistant PI accession(s) listed in the table for a given trial according to a Fisher's LSD test.

^f * = the mean root disease severity value of the control (PII66159 in this case) is significantly greater ($P < 0.05$) than only one of the *Fsp*-resistant PI accession(s) listed in the table for a given trial according to a Fisher's LSD test.

PII84128, PII98735, PI220174, PI220189, PI222071, PI222117 and PI271119 as accessions that consistently demonstrated low RDS values (mean RDS values between 0.92 and 2.32) in two or more screening tests (Table 2). Six of these eight accessions originate from Afghanistan (PII25839, PII98735, PI220174, PI220189, PI222071 and PI222117) and one accession each from China (PI271119) and Yugoslavia (PII84128). Thus, the most *Fsp*-resistant wild pea germplasm based on RDS values is represented by only three countries. The high number of *Fsp*-resistant accessions originating from Afghanistan may indicate a unique source of genetic resistance to *Fsp* that is specific to the wild germplasm from that country. My lab

in collaboration with Dr. Norman Weeden at Montana State University screened a mapping population developed to identify the genes/QTLs (Quantitative trait loci) associated with the observed resistance to *Fsp* in the Afghanistan accession PI220174. Preliminary results from this research indicated that the major QTL associated with *Fsp* resistance in PI220174 overlapped the A locus on linkage group II which is the gene associated with purple flowers (17). The seed of purple-flowered cultivars are not popular for human consumption due to flavor and pigment issues, calling into question the practical use of the Afghanistan *Fsp* resistance if the gene or genes associated with the resistance are the same genes associated with purple-flowered varieties that are currently not marketable for human consumption. However, despite these apparent obstacles, no mapping populations have been developed to assess whether the resistance to *Fsp* originating from PI accessions from China (PI271119) or Yugoslavia (PI184128) are associated with different or similar genomic regions as those observed in the Afghanistan accessions and whether they may be alternative sources of resistance.

Resistance to a pathogen may result in a fitness cost to the host to maintain that resistance. Therefore, the ability of an infected plant to maintain similar growth as that of a non-infected plant grown under the same conditions, is a good indicator of the tolerance of that plant to a particular pathogen. Eight (PI125839, PI125840, PI175226, PI220174, PI223526, PI223527, PI226561 and PI227258) of the 44 accessions screened in this study demonstrated high levels of tolerance to *Fsp*, since the mean height, foliage fresh weight, foliage dry weight and root dry weight of the inoculated plants was not significantly different from non-inoculated plants of the same accessions in repeated tests. Five of these eight accessions originate from Afghanistan (Table 3). The other three accessions originate from Iran (PI227258), Ethiopia (PI226561) and India (PI175226). These tolerant lines may have additional genes

Table 3. PI accession number, mean plant height, foliage fresh weight, foliage dry weight and root dry weight of selective wild pea germplasm from the *Pisum* Core Collection in Pullman, WA, USA of which the seed was either inoculated with *Fusarium solani* f. sp. *pisi* (*Fsp*) or not inoculated (water treated) in repeated tests and plants were assessed 25 days after planting. Germplasm presented in the table represents tolerant accessions to *Fsp* with no significant differences ($P > 0.05$) between the inoculated and non-inoculated seed in repeated tests for all the parameters measured according to a Fisher's LSD test. A total of forty-four accessions were screened.

PI accession	Test	Plant height (cm) ^a	Foliage fresh wt. (g)	Foliage dry wt. (g)	Root dry wt. (g)
125839	1a	11.98/11.95	0.599/0.531	0.098/0.075	0.083/0.083
125839	1b	22.67/25.32	0.850/0.961	0.104/0.105	0.082/0.086
125839	1c	12.20/11.87	0.706/0.679	0.094/0.086	0.146/0.161
125840	1a	15.74 /17.17	0.465/0.542	0.078/0.084	0.067/0.073
125840	1b	29.86/31.91	0.722/0.764	0.090/0.090	0.063/0.056
125840	1c	17.58/15.56	0.643/0.578	0.091/0.073	0.108/0.113
175226	1a	25.54/26.32	0.974/0.995	0.156/0.135	0.094/0.087
175226	1b	42.94/44.59	1.320/1.278	0.151/0.151	0.082/0.073
175226	1c	25.59/24.43	1.125/1.142	0.149/0.139	0.138/0.152
220174	3a	31.95/31.31	0.782/0.800	0.089/0.089	0.074/0.069
220174	3b	28.02/29.10	0.716/0.722	0.091/0.079	0.088/0.076
223526	3a	30.90/30.82	0.754/0.673	0.086/0.078	0.062/0.067
223526	3b	27.80/28.91	0.648/0.676	0.085/0.083	0.076/0.072
223527	3a	28.72/29.11	1.089/0.957	0.114/0.100	0.122/0.089
223527	3b	24.87/27.53	0.893/0.962	0.111/0.112	0.113/0.120
226561	3a	37.27/36.66	1.46/1.401	0.147/0.143	0.116/0.107
226561	3b	35.93/38.86	1.469/1.592	0.186/0.184	0.126/0.132
227258	3a	33.68/35.20	0.687/0.717	0.078/0.082	0.063/0.061
227258	3b	29.85/32.01	0.653/0.696	0.086/0.088	0.066/0.061

^a The first number followed by a "/" is the mean value of the inoculated plants followed by the mean value of the non-inoculated plants for the specified parameter. Seed of inoculated plants and non-inoculated plants of each accession were treated with 1×10^6 macroconidia of *F. solani* f. sp. *pisi* and sterile distilled water, respectively. Roots of non-inoculated plants were free of infection.

that are different from the genes providing partial resistance to *Fsp* root infection that allow them to tolerate root infection by this pathogen while maintaining similar growth as non-infected plants, thus

these plants are not demonstrating a fitness cost for *Fsp*-resistance. The plant growth of PI accessions 125839, 125840 and 175226 in test 1b demonstrated significantly greater growth measurements than in tests 1a or 1c (Table 3). The reasoning for the differential in growth is not understood but could have been due to more favorable greenhouse growing conditions experienced in test 1b.

The mean percent germination of all accessions screened in this study was not significantly different ($P > 0.05$) between the inoculated and non-inoculated seed of the same accessions in two or more screenings in each test, indicating that the 44 accessions were resistant to seed rot or pre-emergence seedling damping off by *Fsp* under our screening conditions. Forty-three of the forty-four accessions screened have pigmented seed coats, with PI244121 (non-pigmented seed) being the only exception. Peas with pigmented seed coats are believed to be more resistant to seed rot or seedling damping off caused by *Fsp* than peas with non-pigmented seed coats (16). This theory was supported by the present results, since the mean percent germination of the inoculated seed of Bolero, DSP and PI244121, all having non-pigmented seed coats, was significantly less ($P < 0.05$) than the non-inoculated seed of these same pea lines in 8 of 8, 10 of 10, and 1 of 2 screening tests, respectively. There were only three incidences among the tests where the mean percent germination of an accession having pigmented seed was significantly less than the non-inoculated seed of the same accession (data not shown).

Future research will look to identify the genes/QTLs identified in this study that confer both partial resistance and tolerance to *Fsp*. A major emphasis will be placed on further evaluation of *Fsp*-resistant genes present in the wild Afghanistan pea accessions in addition to the potential new sources of resistance/tolerance from wild pea germplasm from Yugoslavia, China, Iran, Ethiopia and India. Efforts will be made to develop pea cultivars that will incorporate these resistances/tolerances and be used successfully throughout pea growing regions to manage Fusarium root rot.

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Field quantification of foliar chlorophyll content in *Pisum* germplasm

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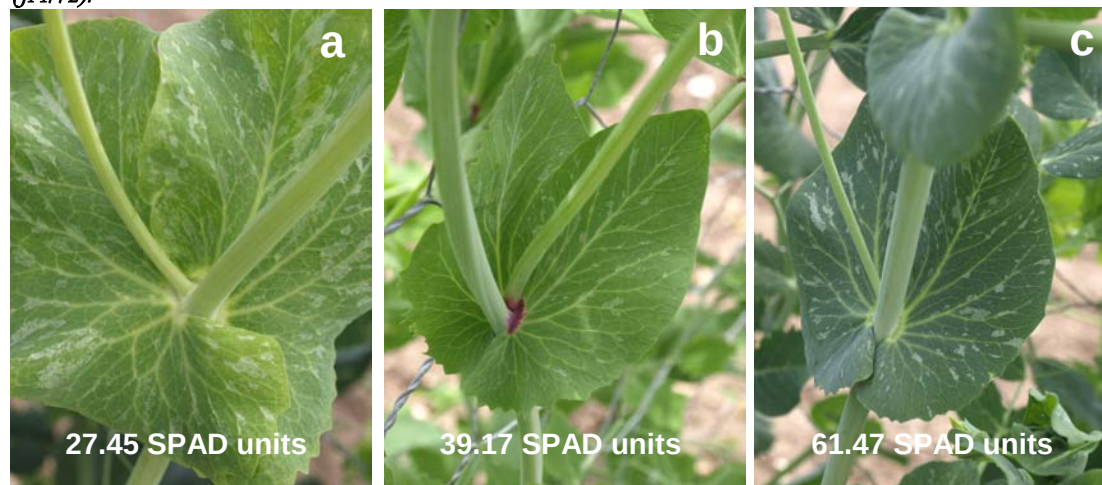
John Innes Centre, Norwich, UK

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 (1), 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 three JI accessions growing in the field. a. *yellow green* (JI 799), b. *green* (JI 1889) and c. *dark green* (JI 1772).



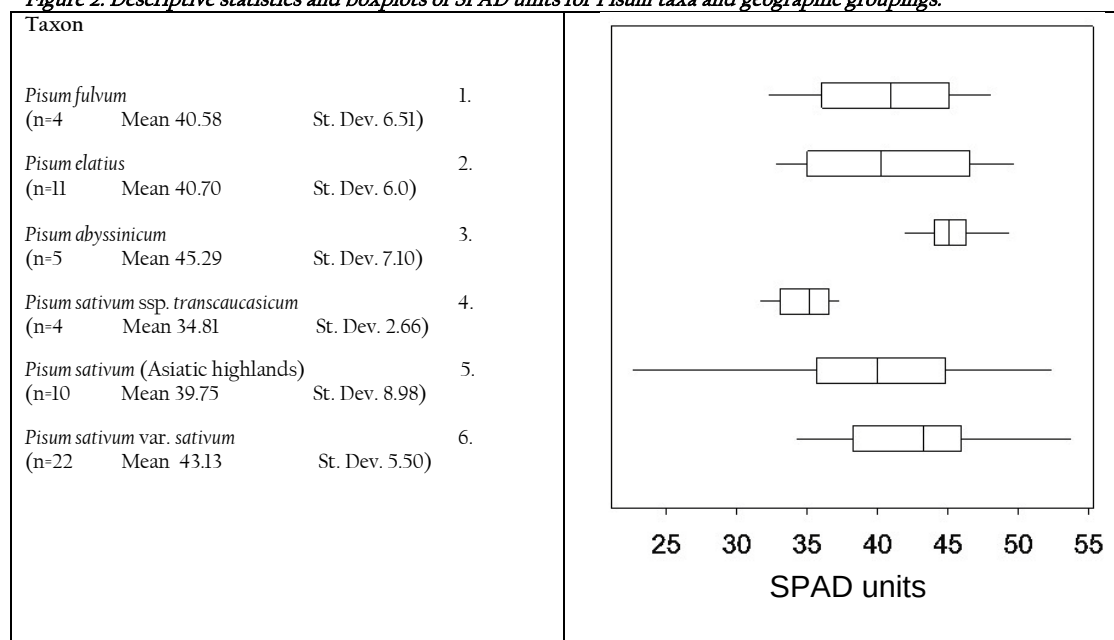
Readings were taken from the same accessions two weeks after the initial set at the end of flowering (end of June). The two sets of readings were very similar with the same levels and differentials between lines evident throughout (data not shown).

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 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.



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 $\pm$ 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 $\pm$ 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 (11, 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 1 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

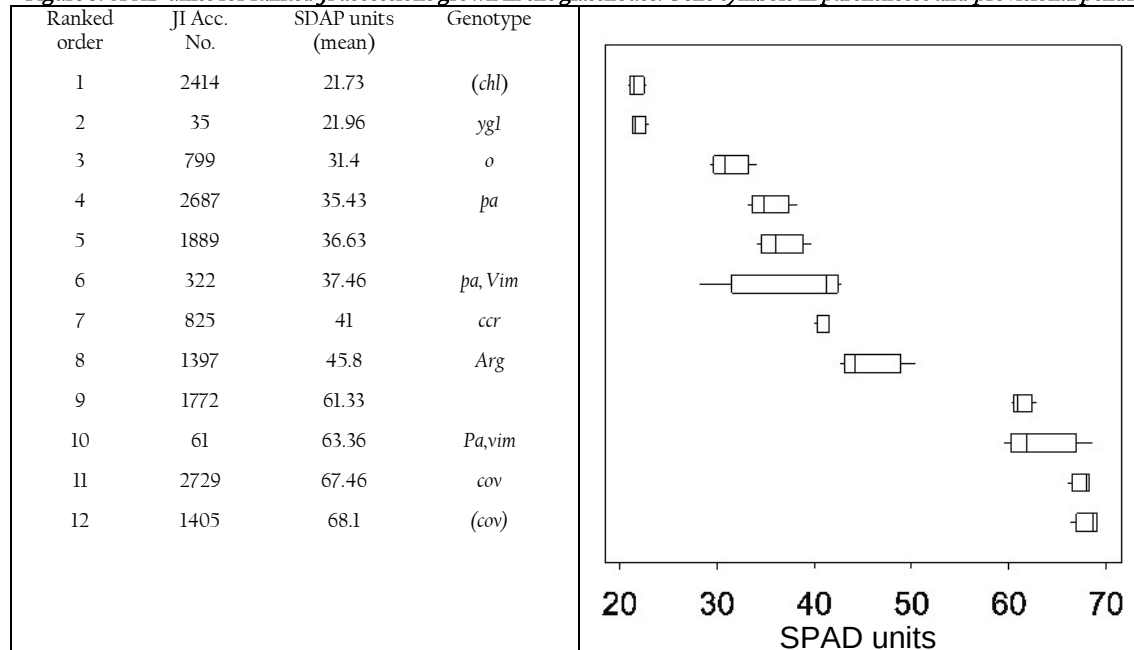
In the autumn a glasshouse survey was undertaken on a range of type lines and mutants for genes associated with foliage chlorophyll content. This included four lines that had been measured in the field earlier in the summer (section 1). For this survey, three readings were taken from separate leaflets (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.

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 Šančevi, 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 ₂ O	CaCO ₃ (%)	Humus (%)	N total (%)	Al-P ₂ O ₅ (mg 100g ⁻¹)	Al-K ₂ O (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 m² 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):

$$LER = Sd_{IC} / Sd_{PS} + Sg_{IC} / Sg_{PS}$$

, 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.

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-leaved 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

Acknowledgements

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Distribution and new records of *Pisum sativum* subsp. *elatius* in Serbia

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The taxon *Pisum sativum* L. subsp. *elatius* (Steven ex M. Bieb.) Asch. & Graebn. (syn. *P. sativum* subsp. *biflorum*) was regarded as a separate species, *P. elatius* (1, 2, 3), and now is commonly accepted as a pea subspecies (4). Based on its long, climbing stems (up to 150 cm), *P. sativum* subsp. *elatius* may be translated into English as *tall pea*. This taxon is also regarded as one of ancestral forms of the cultivated pea, *P. sativum* L. subsp. *sativum* (5).

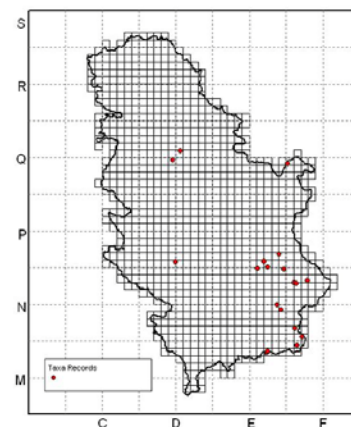
According to its general distribution and ecology, *P. sativum* subsp. *elatius* is a widely dispersed Mediterranean element with native range spreading across the Mediterranean and Oriental-Thuranian floristic regions of the world. More precisely, it is distributed over the coastal region of North Africa (Algeria, Egypt, Libya, Morocco and Tunisia), southwest Asia (Cyprus, Iran, Israel, Lebanon, Syria and Turkey), Caucasus and Crimea (Azerbaijan, Georgia, Russian Federation and Ukraine), southwest Europe (France, Portugal and Spain) and southeast Europe (Italy, Romania and the Balkan Peninsula). The area in southeast Europe that concerns the Balkan Peninsula overlaps mainly with Mediterranean and Sub-Mediterranean parts of Albania, Bulgaria, Croatia, Greece, Macedonia, Montenegro and Serbia (6, 7).

Excluding *P. sativum* subsp. *arvense*, which is doubtfully native (1), *P. sativum* subsp. *elatius* is likely the only wild growing native pea in Serbia (1). *P. sativum* subsp. *arvense* was recorded in the field in the south (8) and is commonly grown as a component of fodder mixtures (with barley or wheat) throughout Serbia (1). *P. sativum* subsp. *elatius* has been considered distinct in Serbia for a long time. The first records of *P. sativum* subsp. *elatius* in Serbia come from the second half of 19th century and belong to Dr. Josif Pančić, a well known Serbian botanist (9). Later literature sources describe it as a widely distributed plant in the flora of Serbia, mostly on untilled land and rather rarely as a cultivated species (1, 10, 11). A special form of *P. sativum* subsp. *elatius*, designed as f. *albiflorum* Beck and with the whole corolla white, was mentioned as appearing sporadically in comparison to the more typical f. *elatius*, with the corolla parts in various hues of pink (1).

In addition to the field survey, herbarium material and numerous literature sources were used to supplement the distribution records of *P. sativum* subsp. *elatius* in Serbia. The distribution is mapped on a 10 x 10 sq. km UTM grid system (UTM Zone 34T). The results were incorporated into the Global Information System (GIS), spatially analyzed and represented in cartographic form. DIVA-GIS 5.2.0.2 software was used for GIS analysis (<http://www.diva-gis.org/>). Nomenclature, synonyms and distribution out of the Balkans are given according to the Germplasm Resources Information Network - (GRIN) database (<http://www.ars-grin.gov/cgi-bin/npgs/html/taxgenform.pl>).

Although it was reported to be widely distributed in Serbia (1), *P. sativum* subsp. *elatius* seems to be more rare or rather regionally threatened as depicted in Figs. 1 and 2. In addition, some of the

Figure 1. Distribution of *P. sativum* subsp. *elatius* in Serbia (UTM, Zone 34T, 10x10).



habitats in the northern part of its area, recorded in the vicinity of Belgrade (9) and referring to the first records in Serbia are seemingly destroyed or even endangered by human activities. According to the data collected, the highest density of *P. sativum* subsp. *elatius* populations is located in the southeast and South Serbia (Fig. 1). Recently, a new population of *P. sativum* subsp. *elatius* was located in the valley of the river Pčinja, in the far southeast of Serbia and near its borders with Bulgaria and Macedonia (Fig. 2). This evidence has implications for the recent activities on the wild and semi-wild *Pisum* taxa in the Balkans, resulting in finding 'tall pea' in several neighboring regions of Bulgaria (2, 12) and Macedonia (13).

The lowest, gorge-like, part of the Pčinja valley is characterized by the presence of a significant number of Mediterranean elements of flora (14) and vegetation that comprise different plant communities. The large area of the previous forest habitats is devastated now and is extremely dry and covered by diverse herbaceous vegetation such as thermophilous grassland vegetation and pseudosteppe formations. In relation to its vegetation and floristic peculiarity the Pčinja valley represents a transition area to the Mediterranean region (Fig. 3). Mediterranean influences penetrate in the valleys of great rivers of the Aegean river basin and radiate along their tributaries via Macedonia up to the mountains in South Serbia (15, 16).

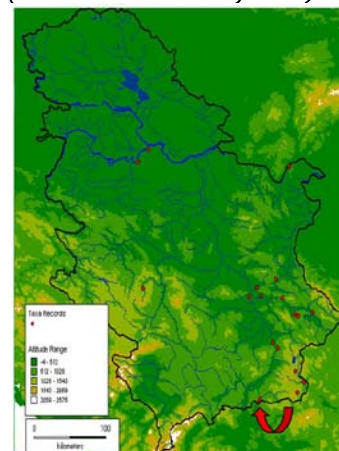
Figure 3. Thermophilous grasslands of Pčinja valley rich in legume species (*Vicia tenuifolia* subsp. *dalmatica*).



subsp. *thuretii*, *Cardamine graeca*, *Moenchia graeca*, *Scleranthus perennis* subsp. *dichotomus*, *Tuberaria guttata*, *Hypericum montbretii*, *Potentilla laciniosa*, *Comandra elegans*, *Astragalus onobrychis*, *Chamaecytisus triflorus*, *Lens nigricans*, *Vicia tenuifolia* subsp. *dalmatica*, *Jasione heldreichii*, *Campanula lingulata*, *Fritillaria gussichiae*, *Iris suaveolens*. Both *Pisum sativum* subsp. *elatius* subpopulations inhabit a relatively small area, restricted exclusively to the gorge of Pčinja, and are included in the Natural Asset "Dolina reke Pčinje" which is protected at the national level as The Landscape of Outstanding Features.

The population of *P. sativum* subsp. *elatius* is not so easily accessible and distant from regularly tilled land, confirming the quotations (6) that this species prefers rocky and grassy slopes, forest and field margins, scrub and ruins, with an altitude of between 0 m and 1700 m. The new *P. sativum* subsp.

Figure 2. Position of *P. sativum* subsp. *elatius* populations in Serbia related to its relief and hydrographic features (new records indicated by arrow).



P. sativum subsp. *elatius* inhabits the lower zone of northerly exposed slopes of Mount Kozjak (UTM square EM-78; Fig. 1) on the left side of the valley. The slopes are covered by thermophilous submediterranean forests and scattered scrub vegetation consisted of pubescent oak (*Quercus pubescens*) and prickly juniper (*Juniperus oxycedrus*) overgrowing siliceous rocky ground. Two small groups of the individuals were recorded in the edges of the forests, in open spaces between the trees and shrubs, and on the sides of paths through the foothills (Fig. 4). A wealth of flowering herbaceous plants were in full bloom in May. The following plant list, though not complete, indicates the floristic and ecological flora which occur most commonly with tall pea: *Fumaria petteri*

Figure 4. A *P. sativum* subsp. *elatius* plant from the habitat in the valley of Pčinja (photo by B. Zlatković).



elatus population is temporarily protected from the risk of loss by grazing or some other similar danger related to the activity of man (Fig. 4, Fig. 5).

The future activities for both *in situ* and *ex situ* conservation of *P. sativum* subsp. *elatus* are scheduled for 2011 and will comprise a more detailed study on its population in the valley of Pčinja, as well as the expeditions aimed at discovering the new populations in the wider region of southeast Serbia. Significant improvement in the characterization and evaluation of the populations is planned, especially of the traits of agronomic importance such as forage and seed yield and stress tolerance. The threatened status of *P. sativum* subsp. *elatus*, according to the IUCN Red List Categories and Criteria (17) and relevant national legislatures of Serbia should present useful information for plant protection management in the region.

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Figure 5. *P. sativum* subsp. *elatus* from the new recorded population, a detail with flowers (photo by B. Zlatković).



Potential alternative hosts for the pea powdery mildew pathogen *Erysiphe trifolii*

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Powdery mildew is an important disease of peas grown in both greenhouses and in the field. The latter is obviously important for commercial production, but greenhouses are often used to increase the number of generations per year in pea breeding programs. Even though *Erysiphe pisi* (often reported as *E. communis* or *E. polygoni* in earlier literature) is the most commonly documented pathogen species causing powdery mildew of peas, *E. baeumleri* (1) and *E. trifolii* (2) were recorded as powdery mildew pathogens on peas. It has been presumed that the putative breakdown of resistance in previously resistant pea cultivars observed in the US Pacific Northwest (US PNW) was actually due to the presence of more than one species of *Erysiphe* (2). Attanayake et al. (2) observed severe disease symptoms caused by *E. trifolii* on resistant pea cv. 'Lifter' grown in greenhouse conditions. Greenhouse-grown pea breeding materials often get infected with powdery mildew in the US PNW (K. McPhee, personal communication). However, the inoculum source, particularly for greenhouse-grown peas during the winter months, has not been determined. Since during winter months no pea crops are growing in fields in the PNW, inoculum would have to originate from pea debris of the previous growing season, volunteer pea plants or from powdery mildew-infected wild legume plants serving as alternative hosts. Many powdery mildew pathogens are known to have broad host ranges (3). *E. trifolii* has been reported on peas and lentils in the US PNW (2, 4) and on *Trifolium* (as the specific epithet indicates) and other genera of the Fabaceae such as *Acacia*, *Arachis*, *Lathyrus*, and *Melilotus* (5). Species of *Arachis*, *Dolichos*, *Lathyrus*, *Lens*, *Lupinus*, *Medicago*, *Melilotus*, *Phaseolus*, *Trifolium* and *Vicia* are known hosts for *E. pisi* (5). The above abbreviated host lists make it clear that *E. trifolii* and *E. pisi* have numerous hosts, including some hosts in common.

Powdery mildew-infected *Medicago lupulina*, *Melilotus* spp., *Lathyrus* spp. and *Vicia* spp. plants are abundant along road sides, recreational areas and commercial fields during the periods of July-November in the Palouse region of Idaho and Washington. We hypothesized that these weedy legumes can serve as alternative hosts for *E. trifolii*. Detailed studies on host range of *E. trifolii* in the US PNW are lacking, so we tested these common weedy legumes from the region as potential alternative hosts of *E. trifolii*.

Materials and Methods

Plant germplasm

To assess susceptibility to *E. trifolii*, we obtained germplasm (seeds) from the USDA-ARS National Plant Germplasm System for nine wild legume species: *Medicago lupulina* L. (PI189128), *Medicago scutellata* (L.) Mill. (PI161415), *Melilotus albus* Medik. (PI90186), *Melilotus officinalis* L. (PI539020), *Medicago polymorpha* L. (PI186329), *Lathyrus latifolius* L. (PI358888), *Trifolium pratense* L. (PI631906), *Vicia amoena* Fisch. (PI428330), and *Vicia cracca* L. (PI371785), all from the North Central Plant Introduction Station at Ames, IA. *Vicia faba* L. seeds were obtained from Mountain Valley, Inc. in Salt Lake City, UT. To test cultivated legumes as potential alternative hosts, seeds of powdery mildew-susceptible *Glycine max* (L.) Merr. (cvs. L84-2237 – PI547870 and Harosoy- PI548573) seeds were obtained from the USDA Soybean Germplasm Collection in Urbana, IL and seeds of susceptible *Lens culinaris* Medik. cultivar 'Crimson' were obtained from the USDA Grain Legume Genetics and Physiology Research Unit, Washington State University (WSU), Pullman, WA. *Glycine max* cultivars, L84-2237 – PI547870 and Harosoy- PI548573 are susceptible to *E. diffusa* (6, 7).

This latter powdery mildew species was of interest, since a powdery mildew reported from lentil in Canada was identified as *E. diffusa* on the basis of morphology (8).

To maintain powdery mildew inoculum, seeds from four powdery mildew-susceptible pea cultivars; 'Dark Skin Perfection', 'Medora', 'Radley' and a previously registered powdery mildew resistant cv. 'Lifter' (9) were obtained from USDA-ARS Grain Legume Genetics and Physiology Research Unit, WSU, Pullman, WA.

Greenhouse inoculation

All the inoculations were carried out at the WSU Plant Growth Facility. Pea seeds of all varieties were grown in the greenhouse and allowed to get natural infections to serve as inoculum for all the experiments. Once pea plants became infected, the pathogen was identified by isolation of DNA from colonies of powdery mildew and sequencing the rDNA ITS 1 and ITS 2 regions as described by Attanayake et al. (4). Sequences were compared with those in GenBank. Powdery mildew conidia were collected from 3-4 plants of each pea cultivar and ITS sequencing was determined separately (by cultivar) in order to identify the pathogen species. Morphological characters of the pathogen were observed under a compound microscope and diagnostic characters of chasmothecia, if present, were documented.

Seeds of each of the above mentioned plant species were planted in 15cm pots. Plants were kept side by side in the same greenhouse with powdery mildew-infected pea plants. Twenty-four seeds of each plant accession were planted using six replicate pots with four seeds per pot. Three pots of each species served as controls and the rest of the plants were artificially inoculated. Inoculations were carried out when plants were 21 days old. Powdery mildew conidia were collected in a plastic weighing boat and carefully dusted onto the adaxial surfaces of the first six fully expanded younger leaves until white colored conidia were visible on the surfaces. Symptom development was observed every two days after inoculation until the flowering stage.

Results

All pea cultivars exhibited severe powdery mildew symptoms. Amplified PCR products were 650 bp in size. BLAST search results showed that the ITS sequences were identical to one another and 99% similar to *Erysiphe trifolii*. 97% similarity was exhibited to *E. pisi* deposited by Saenz and Taylor (10). Severe powdery mildew infections were produced on the pea cv. 'Lifter', a cultivar previously reported to be resistant to powdery mildew caused by *E. pisi*. Chasmothecia were observed on all pea cultivars and appendages were long, flexuous and dichotomously branched, typical of an authentic specimen of *E. trifolii* and isolates previously collected in the US PNW (2).

In the greenhouse inoculation studies, powdery mildew colonies were observed on all plants tested at 10-14 days after inoculation, except in the case of *Glycine max* on which no colonies were observed. Those soybean genotypes were previously known to be susceptible to *E. diffusa* (6, 7). Due to the high disease pressure all control plants, except those of *G. max*, ultimately became infected within 15-18 days after the inoculation date.

Discussion

Results of cross inoculation studies conducted in the greenhouse showed that *Lathyrus latifolius*, *Medicago polymorpha*, *M. lupulina*, *M. scutellata*, *Melilotus albus*, *M. officinalis*, *Trifolium pretense*, *Vicia amoena*, *V. cracca*, and *V. faba* are potential alternative hosts for the pea powdery mildew pathogen, *E. trifolii*, and confirmed our previous reports that *Lens culinaris* and *Pisum sativum* are also hosts (2, 4). Our previous cross inoculation studies using a detached leaf bioassay showed that conidia of *E. trifolii* collected from *Melilotus albus* and *Lens culinaris* caused powdery mildew on *Pisum sativum* and conidia of *E. trifolii* collected from *Pisum sativum* caused powdery mildew on *Melilotus albus* and *Lens culinaris* (2, 4), which further supports the conclusions

of the current study. The lack of any symptom development of powdery mildew on the soybean cultivars reported to be susceptible to *E. diffusa* indicates that the powdery mildew species in this study was not *E. diffusa* and soybean is resistant to *E. trifolii*.

The findings of this study broaden the understanding of the wide host range of powdery mildew pathogens infecting peas. Findings are particularly pertinent to weed management, screening for powdery mildew resistance, provide information for consideration in developing strategies to select for resistance in pea breeding programs and to manage powdery mildew of pea in the field.

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Contributions to the characterization of *Vavilovia formosa* (syn. *Pisum formosum*). I. Anatomy of stem, leaf and calyx

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It was recently reported that *Vavilovia formosa* Stev. Fed., informally referred to as *beautiful vavilovia* or simply *vavilovia*, has been gaining interest since its importance for legume molecular taxonomy and potential use in breeding as a useful crop wild relative (1). This is not surprising since it is a relict and endangered species limited to the Caucasus and its adjacent regions (2). As the only species of the genus *Vavilovia* Fed., it is often considered the closest to the common ancestor of the tribe *Fabeae* (syn. *Vicieae*) (3), comprising vetchlings (*Lathyrus* L.), lentils (*Lens* Mill.), peas (*Pisum* L.) and vetches (*Vicia* L.).

V. formosa is a perennial herbaceous species, with a dwarf habit and plant height between 5 and 15cm. Its stems are slender, sprawling or creeping, not winged and with a glabrous surface. The leaf of *V. formosa* is compound as in all other *Fabeae* with small, semi-sagittate and foliaceous stipules, one pair of broadly, cuneate-obovate to suborbicular, thick and glabrous leaflets with non-indentated margins and the rachis ending with a mucro-like formation similar to faba bean (4).

Little is known about the anatomy of vegetative and reproductive organs of *V. formosa*. The aim of this preliminary research was to gain knowledge of the anatomical characteristics of *V. formosa* in order to properly understand the biology, ecology, adaptation strategy and nutritive value of this species.

Materials and Methods

On July 17, 2009, the first of the three expeditions was carried out to the Mount Ughtasar region in southern Armenia aimed at *in situ* research and *ex situ* conservation of *V. formosa* in Armenia. The Mount Ughtasar region is one of the three main habitats of *V. formosa* in Armenia (5). During the expedition, samples of stems, leaves and flowers of one population growing at an altitude of between 3305 and 3315 masl were collected. The collected fresh plant material was fixed in Carnoy I, a 3:1 solution of ethanol and glacial acetic acid.

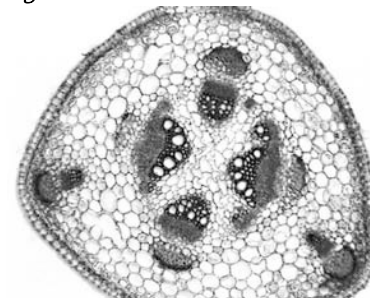
The anatomical structures of stems and leaves, including the structure of leaflets, stipules, petiole and the rachis tip were observed using light microscopy and an Image Analyzing System, Motic 2000 (Motic, Wetzlar, Germany). Analysis of the calyx was added since fresh and properly fixed floral material was available. Cross sections of both aboveground vegetative organs and the calyx were made using a Leica CM 1850 cryostat (Leica, Solms, Germany) at a temperature of -18 to -20 °C and at cutting intervals of 25 µm.

Results

Stem. The stem is rounded in cross section, with two small wings, which are not prominent (Fig. 1). The primary cortex is well developed, with two layers of chlorenchyma cells and several layers of parenchyma cells without chloroplasts. No collenchyma is recorded. Two cortical vascular bundles, one in each wing,

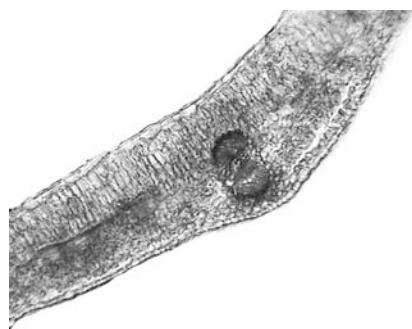
also occur. In the central cylinder, collateral open vascular bundles of different size are arranged in a circle. Only small groups of sclerenchyma are present near the bundles. Sclerenchyma cells adjacent to the phloem have larger lumen and less lignified walls. Crystals occur in parenchyma sheath cells above the sclerenchyma strands. The central cylinder is composed of parenchyma with no central cavity or sclerenchymatic parenchyma present. Parenchyma cells of the cortex and central cylinder sometimes contain starch grains.

Figure 1. *V. formosa* stem cross section



Leaflet. The leaflets of *V. formosa* have dorsiventral structure (Fig. 2). The epidermis has a single layer composed of cells with sinuous anticlinal walls and covered with a thick cuticle. It contains large numbers of stomata on both the adaxial and abaxial surface. Stomata are slightly sunken or at the same level as other epidermal cells. The leaves are glabrous on the adaxial side, but rare non-glandular trichomes were observed abaxially along the leaf veins. Leaf photosynthetic tissue is differentiated on palisade and spongy tissue. Palisade tissue is well developed, composed of two to three layers of elongated cells. Under the abaxial epidermis 4-5 layers of spongy tissue cells of irregular shape were observed. Mesophyll cells were closely arranged with small intercellular spaces. Closed collateral vascular bundles were arranged in a single row. Parenchyma sheath cells along the vascular bundles had solitary prismatic crystals. They are larger by the vascular bundle in the main vein which is not very prominent on the abaxial side. Collenchyma tissue is not present or only a few collenchyma cells occur abaxially.

Figure 2. *V. formosa* leaflet cross section



Stipule. Stipules have anatomical structure very similar to the structure of the leaves (Fig. 3). Palisade tissue is not as dominant as the one in the leaves. It is composed of one or two layers of cells. Spongy tissue is present in three to four layers. Larger groups of sclerenchyma tissue are recorded by vascular bundles with solitary crystals in the sheath cells. Stipules are also glabrous.

Figure 3. *V. formosa* stipule cross section

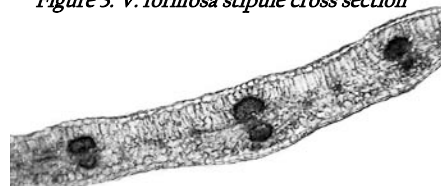
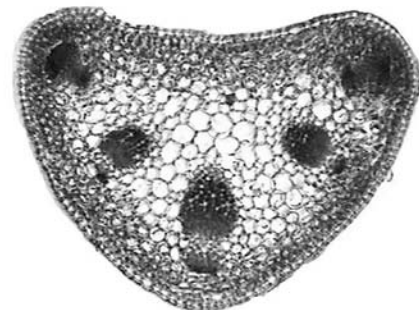


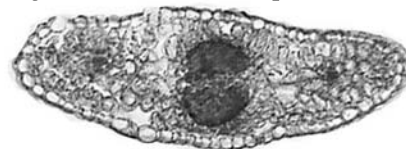
Figure 4. *V. formosa* petiole cross section



Petiole. The petiole is triangular or heart-shaped in cross section (Fig. 4). The epidermis has a single layer with sparse non-glandular trichomes. Four to five layers of chlorenchyma tissue occur subepidermally. Collenchyma is present in small groups only in the main rib. Five collateral vascular bundles are arranged in the form of an open arc. Sometimes, three vascular bundles in the main ribs are much larger than the other two. Small groups of sclerenchyma tissue occur by the phloem and are somewhat larger by the xylem. Solitary crystals were observed in parenchyma sheath cells above the vascular bundles. The central part of the petiole is filled with parenchyma cells. The central cavity is not present.

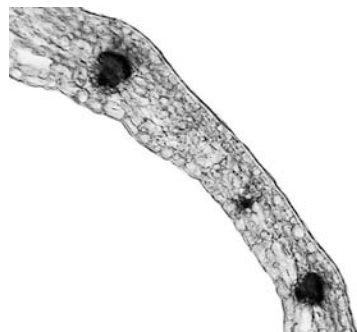
Rachis tip. The cross section of a *V. formosa* rachis tip is elliptical in shape (Fig. 5). On the adaxial side one layer of palisade cells is present. Mesophyll is compact with densely distributed cells.

Figure 5. *V. formosa* rachis tip cross section



Three vascular bundles, one large in the middle and one smaller on each side, are present in the mesophyll. Sclerenchyma tissue is recorded by the phloem and xylem and crystals are visible in vascular bundle sheath cells.

Figure 6. *V. formosa* calyx cross section



Calyx. The calyx has an egg-like shape in cross section (Fig. 6). A thick cuticle covers the outer epidermis. Non-glandular and glandular trichomes are present on the inner epidermis. Non-glandular trichomes are thin, long, composed of one basal and elongated terminal cell. Three types of glandular trichomes are detected: Type I with one stalk cell and elongated multicellular secretory head, Type II with one stalk cell and a two-celled secretory head and Type III with a two-celled stalk and two-celled secretory head. Mesophyll was composed of 3-5 (or 7) layers of cells. Ten larger vascular bundles and several smaller between them are arranged in a circle. Crystals are present in parenchyma cells adjacent to the vascular bundles.

Discussion

The results of anatomical analysis showed that *V. formosa* has general anatomical structure that is characteristic for most of the plants of family Fabaceae (6). However, this species developed some anatomical modifications, according to the environmental conditions of the specific habitat where it grows. It inhabits high mountain areas and grows on slopes with sparse vegetation coverage (5, 7). The slopes with *V. formosa* populations are sun-warmed and exposed to sunlight. The plant organ that reacts most to the environmental factors, especially the amount of sunlight, is the leaf (8). The leaves of *V. formosa* had helio-xeromorphic structural adaptations. They were relatively thick, covered with a thick cuticle, with slightly sunken stomata and with strongly developed palisade tissue. Furthermore, palisade tissue cells were elongated, narrow and arranged in three rows, which were adaptations that enabled this species to survive in this specific habitat.

According to the previous morphological descriptions of the *V. formosa* stem, it is slender, sprawling or creeping and without wings (5, 9). However, anatomical investigations of stem structure showed that stems had lateral wings which were not prominent but contained one cortical vascular bundle each. These wings were hard to observe by morphological examination only; they looked more like small stem ribs, but on cross sections the presence of wing bundles was obvious. The presence of a creeping stem, assigned as thread-like rhizoma by some authors (7), could be explained by the fact that stem mechanical tissue was not well developed. Collenchyma tissue was not recorded and sclerenchyma cells formed small groups by the vascular bundles. Sclerenchyma cells adjacent to phloem had larger lumen and less lignified cell walls. This amount and pattern of distribution of mechanical tissue could not provide enough mechanical support to the stem to keep it in an upright position (10).

It was noticed that *V. formosa* had been frequently grazed by wild and domestic animals, which was one of the main problems for its conservation (5, 7). One of the reasons for intensive grazing may lie in the fact that all analyzed vegetative plant parts are practically glabrous, contain small amounts of indigestible mechanical tissue and large amounts of well developed parenchyma tissue, which are favorable forage characteristics for animal consumption. These anatomical features of *V. formosa* should be further analyzed as potential benefits that could be introduced to other cultivated *Fabaceae* plants and close relatives of this wild-growing species.

Acknowledgements

This work was financially supported by the Ministry of Science and Technological Development, Republic of Serbia, Grants No. and 20090 and 31024.

The authors are grateful to Darko Znaor, Zvonimir Bede and Dušan Dozet for their assistance.

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Contributions to the characterization of *Vavilovia formosa* (syn. *Pisum formosum*). II. Morphology of androecium and gynoecium and mitosis

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Vavilovia (*Vavilovia formosa* Stev. Fed., syn. *Pisum formosum*) is the only species of the genus *Vavilovia* Fed. included in the tribe *Fabeae* with vetchling (*Lathyrus* L.), lentil (*Lens* Mill.), pea (*Pisum* L.), and vetch (*Vicia* L.) (1). Largely ignored for decades, *V. formosa* has become the object of recent attention by geneticists and molecular taxonomists (2) due to its specific position within the tribe and the supposed role in its evolution (3).

The flowers of *V. formosa* are often solitary, axillary and pedunculate, with small and/or inconspicuous bracts, lacking bracteoles, and having a campanulate calyx. The corolla of *V. formosa* is pink or purple with an oblong standard, falcate to oblong wing and blunt, non-cristate and sometimes white keel (4).

This preliminary research was aimed at examining the morphology of the reproductive organs of *V. formosa* with emphasis on the androecium and gynoecium, as well as understand some basic cytogenetics of the species.

Materials and Methods

The first of the three expeditions aimed at *in situ* research and *ex situ* conservation of *V. formosa* in Armenia was carried out to the Mount Ughtasar in southern Armenia, on July 17, 2009 (5). During this expedition, samples of stems, leaves and flowers of one population growing at an altitude of between 3305 and 3315 masl were collected and fixed in Carnoy I, a 3:1 solution of ethanol and glacial acetic acid.

The parts of *V. formosa* flowers were observed using stereo microscope Stereo microscope Stemi 2000 (Carl Zeiss, Göttingen, Germany) and photographed by the Power Shot G5 Digital Camera (Canon, Japan). The pollen grains were removed from the anthers and their viability was determined using the staining method of Alexander (6). The youngest leaves from the fixed material were used to determine the chromosome number using the acetocarmine method (7).

Results and Discussion

Androecium. As in many other legume species, the androecium in *V. formosa* is diadelphous, where nine filaments are fused together and anthers are spherical and two-pieced (Fig. 1). In some fully developed flowers only one or two filaments had anthers. In the case of fully developed flowers with regular

Figure 1. Stamens of *V. formosa* enveloping pistil (lower right corner).

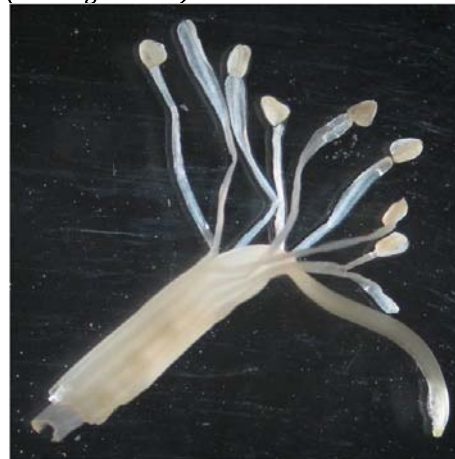
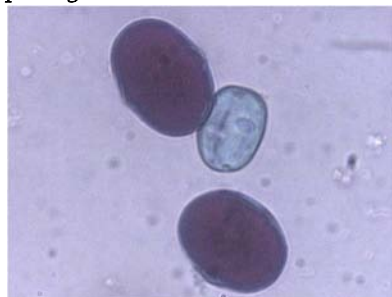


Figure 2. Two fertile and one sterile pollen grains of *V. formosa*.



size and color this could be an indicator that, for unknown reasons, *V. formosa* produces less pollen and; therefore, fewer seeds. The pollen grains of *V. formosa* are ellipsoid-shaped with a thin exine and one pore. The fertile pollen grains are larger than those that are sterile which are often deformed (Fig. 2). On average, the viability of the *V. formosa* pollen grains was 93.3%.

Figure 3. A *V. formosa* pistil



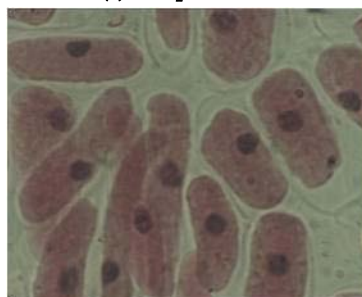
Gynoecium. The ovary in *V. formosa* is protected by the merged calyx. It has a horizontal position while the style and the feather-shaped and hairy stigma have a vertical position that follows the position of a flower (Fig. 3). The ovary usually contains 7 or 8 spherical ovules (Fig. 4), which is more than the 3 to 5 seeds produced from a typical pod (8).

Figure 4. A dissected *V. formosa* ovary with 7 ovules



Mitosis. A large majority of the *V. formosa* cells in the acetocarmine preparations were in interphase (Fig. 5a). Several cells were in metaphase (Fig. 5b) and in anaphase (Fig. 5c), demonstrating that the *V. formosa* somatic cells contain 14 chromosomes as determined earlier by rather rare cytogenetic research on this species (9).

**Figure 5. Mitosis in *V. formosa*:
(a) interphase;**



(b) metaphase;



(c) anaphase.



Conclusions

The obtained results of this preliminary research will hopefully encourage further investigations of the flower apparatus of *V. formosa*, thus contributing to both its more detailed taxonomical description and determining its mating system that may prove helpful for its *in situ* and *ex situ* conservation.

Acknowledgements

This work was financially supported by the Ministry of Science and Technological Development, Republic of Serbia, Grants No. and 20090 and 31024.

The authors are grateful to Darko Znaor, Zvonimir Bede and Dušan Dozet for their assistance.

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Contributions to the characterization of *Vavilovia formosa* (syn. *Pisum formosum*). III. Contents of macro- and microelements

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Vavilovia (*Vavilovia formosa* (Stev.) Fed., syn. *Pisum formosum* (Stev.) Alef.) is a close botanical relative to vetchling (*Lathyrus* L.), lentil (*Lens* Mill.), pea (*Pisum* L.) and vetch (*Vicia* L.) which collectively form the tribe *Fabeae* (1). Its exact taxonomic position within the tribe has been examined for almost two centuries with molecular tools introduced rather recently and with already clarifying outcomes (2).

V. formosa has limited geographical distribution, ranging from the West Taurus Mountains in Turkey to southwest Iran and from Lebanon to north Caucasus. It is a perennial and a typical high-mountain plant (3) that is found at altitudes from 1500 to 3200 masl. *V. formosa* prefers shale or rocky ground, especially loose limestone screes and; therefore, has a well-developed root system (4). The goal of this preliminary research was to assess the chemical composition of the vegetative and generative organs of *V. formosa*.

Materials and Methods

Two expeditions were carried out in 2009 to Mount Ughtasar in southern Armenia which is one of the three main habitats of *V. formosa* in the country (6). The first expedition, on July 17, resulted in collection of herbarium material of roots (Fig. 1a), stems (Fig. 1b), leaves (Fig. 1c) and flowers (Fig. 1d) from one of the *V. formosa* populations, located at an altitude of between 3305 and 3315 masl, and a detailed soil chemical analysis was performed (Table 1). The second expedition to the same place, on August 27, provided pods (Fig. 1e) with mostly immature and not fully developed seeds.

Figure 1. *V. formosa* material collected from the Mount Ughtasar in July and August 2009: (a) roots; (b) stems; (c) leaves; (d) flowers; (e) pods with seeds.



Table 1 Physical and chemical composition of aqueous extract of the soil under a population of *V. formosa* on the Ughtasar Mountain (6).

pH	Saline %	(% / mg-eq. in 100 g of soil)						
		CO ₃	Total CO ₃	Cl	SO ₄ ²⁻	Ca ²⁺	Mg ²⁺	Na ⁺ + K ⁺
7.7	0.118	-	0.039 - 0.64	0.0034 - 0.96	0.008 - 0.17	0.010 - 0.24	0.003 - 0.24	0.024 - 1.03
Carbonate content				CaCO ₃ - 1.3 %; MgCO ₃ - 1.3 %				
Exchange of ions in the clay-humus complex (mg-eq/100 g)				Ca - 16.5; Mg - 4.5 l; Na - 0.4; K - 0.4				
Absorption capacity				21.8 mg-eq/100g				
Mechanical composition				light loamy soil				
Nutrient supply (mg/100g)				N - 2.7 (poor); P ₂ O ₅ - 3.8 (average); K ₂ O - 35.3 (high); humus (%) - 4.4				
Heavy metal mobile forms (mg/kg of humus)				Cu - 5 mg/kg; Ni - 15 mg/kg; Cr - 7 mg/kg; Pb - 6.1 mg/kg; Co - 4.1 mg/kg				

Prior to analysis all plant organs were air dried to a constant moisture content (10%). Total concentration of carbon (C), nitrogen (N) and sulfur (S) in the plant organs were determined by dry combustion using an automatic CNS analyzer (Vario EL III, Elementar). The total concentration of calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), molybdenum (Mo), cobalt (Co) and boron (B) in the root, stem, leaf and flower samples were determined by an ICP-OES (Varian, Vista-Pro) after digestion in a mixture of 10 ml of HNO₃ (65%) and 2 ml of H₂O₂ (30%) using the microwave technique. The concentrations of these elements were not measured in the pods and seeds due to a lack of material.

Results and Discussion

Macroelements. The nitrogen content of *V. formosa* dry matter ranged from 1.37% in the pods to 5.73% in the seeds (Table 2). Crude protein content in leaf dry matter surpasses 200 g kg⁻¹ based of the nitrogen content and the multiplication coefficient of 6.25. Crude protein content of the seed was higher than 350 g kg⁻¹, although the analyzed seeds were not fully mature and thus might contain more crude protein in comparison to mature seeds. Both values may explain why *V. formosa* is severely endangered by grazing from domestic and wild animals and through seed loss caused by mice (5). The carbon content was relatively homogenous in all *V. formosa* organs, varying between 39.97% in its roots and 41.39% in its pods. The flowers and the seeds of *V. formosa* were richest in sulphur with 0.35% and 0.34%, while the stems were the poorest with 0.14%.

V. formosa flowers had the greatest content of Ca (11.40%) (Table 2). Leaves and flowers had roughly twice the content of K (1.44 % and 1.33%) and Mg (0.29 % and 0.27%) compared to roots and stems. The highest Na content was in the flowers (0.13%).

Table 2. Content of macroelements (%) in *V. formosa* organs on a dry matter basis. Values are means $\pm$ SE (n = 3).

Organ	Macroelement content						
	N	C	S	Ca	K	Mg	Na
Roots	1.92 $\pm$ 0.005	39.9 $\pm$ 0.009	0.161 $\pm$ 0.001	0.572 $\pm$ 0.002	0.582 $\pm$ 0.003	0.139 $\pm$ 0.003	0.071 $\pm$ 0.001
Stems	2.25 $\pm$ 0.007	40.4 $\pm$ 0.098	0.139 $\pm$ 0.002	0.826 $\pm$ 0.005	0.742 $\pm$ 0.004	0.156 $\pm$ 0.003	0.098 $\pm$ 0.001
Leaves	3.53 $\pm$ 0.008	40.2 $\pm$ 0.104	0.271 $\pm$ 0.002	2.764 $\pm$ 0.004	1.436 $\pm$ 0.005	0.285 $\pm$ 0.004	0.062 $\pm$ 0.002
Flowers	4.88 $\pm$ 0.003	40.6 $\pm$ 0.056	0.348 $\pm$ 0.004	11.40 $\pm$ 0.046	1.329 $\pm$ 0.003	0.274 $\pm$ 0.003	0.134 $\pm$ 0.002
Pods	1.37 $\pm$ 0.002	41.4 $\pm$ 0.047	0.203 $\pm$ 0.004	/	/	/	/
Seeds	5.72 $\pm$ 0.002	40.9 $\pm$ 0.059	0.338 $\pm$ 0.007	/	/	/	/

Microelements. The content of Cu, Co and Mo in all analysed plant organs were below detection limits. The highest content of Fe (1651 mg kg⁻¹) was in the roots. The roots also had the greatest content of Mn (61.76 mg kg⁻¹) while the leaves had the greatest content of Zn (72.11 mg kg⁻¹). It is noteworthy that boron was accumulated to the highest extent (262.60 mg kg⁻¹) in the flowers of *V. formosa* (Table 3.).

Table 3. Contents of microelements (mg kg⁻¹) in the *V. formosa* organs dry matter. Values are means $\pm$ SE (n = 3).

Organ	Microelement content			
	Fe	Mn	Zn	B
Roots	1651 $\pm$ 9.81	61.76 $\pm$ 0.45	36.77 $\pm$ 0.18	39.60 $\pm$ 0.11
Stems	598.6 $\pm$ 2.03	31.74 $\pm$ 0.24	69.35 $\pm$ 0.08	88.71 $\pm$ 0.22
Leaves	309.9 $\pm$ 0.64	41.84 $\pm$ 0.36	72.11 $\pm$ 0.13	94.72 $\pm$ 0.16
Flowers	157.4 $\pm$ 0.55	42.99 $\pm$ 0.20	52.86 $\pm$ 0.17	262.60 $\pm$ 3.15

Conclusions

The preliminary research on the macro- and microelement content in *V. formosa* provides a solid background for further testing of its tolerance to the lack and surplus of individual elements, as well as assessing its nutritional value and comparison to pea and other related species.

Acknowledgements

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The authors are grateful to Darko Znaor, Zvonimir Bede and Dušan Dozet for their assistance.

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On intraspecific variation of *Vavilovia formosa* (Stev.) Fed. (= *Pisum formosum* (Stev.) Alef.: Fabeae)

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The phylogeny and systematics of tribe Fabeae Rchb. are still somewhat unclear. One of the most enigmatic genera within this group is *Vavilovia* Fed. representing small highland plants with very specific features. It inhabits disrupted areas in Caucasus and Middle East and is commonly referred to as an endangered plant species. Numerous research reports dealing with its morphology, anatomy and taxonomical position (evidenced from results of both classical and molecular analyses) exist (1, 6 and works cited in reviews of these papers). Expressed interest in this genus is evidenced by the appearance of several publications during the last few years (7, 8, 9 etc.). Exhaustive surveys on the history of investigations on this plant species have recently been published (6, 7).

Since this genus was described by C. Steven in 1812 (see 7 for details), numerous data on its intrageneric differentiation were reported. Table 1 summarizes the history of microsystematics of *Vavilovia* with different synonyms listed. The present work together with (11) represent efforts to reveal any intrageneric variation within *Vavilovia* (regardless of whether we treat it as a *Pisum* species or as separate genus).

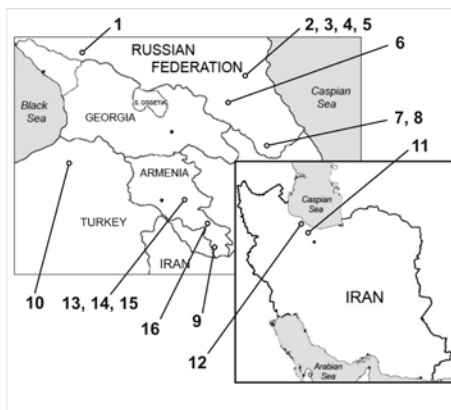
Table 1. Correspondence between different synonyms of intraspecific taxa within *Vavilovia*.

Source		
(4)	<i>Pisum formosum</i> var. <i>typicum</i> Gov.	<i>P. formosum</i> var. <i>microphyllum</i> Ser.
(2)	<i>Vavilovia formosa</i> (Stev.) Fed.	<i>V. aucheri</i> Fed.
(3)	<i>V. formosa</i> (Stev.) Fed.	
(5)	<i>Alophotropis formosa</i> (Stev.) A. Grossh.	<i>A. aucheri</i> (Jaub. & Spach) A. Grossh.

Materials and Methods

The morphometric analysis was performed using photo images of herbarium specimens (MW!, MHA!, ERE!, WIR!). In total 22 specimens were analyzed and 412 leaflets measured. The chosen samples represented collections of different years (1860-2007) from territories of the Kuban' region, Daghestan, Armenia, Turkey and Iran; for details see Figure 1 and Table 2.

Figure 1. Map depicting collection sites of herbarium samples. Numbers correspond to those in Table 2.



All samples were photographed or scanned. The leaves were measured with reference to two parameters, longitudinal and bilateral asymmetry of the leaflet, viz. distance between leaflet base and position of maximum width (l_p), distance between leaflet tip and position of maximum width (l_d), maximum distances from midvein to leaf margin from basiscopic (h_b) and acroscopic (h_a) sides (Fig. 3B). All measurements were performed using Measure™ 2.0 program (C Thing Software). The obtained data were analyzed with the Statistica 8 (Statsoft Inc., Tulsa, OK, USA) software package.

Table 2. List of herbarium specimens used for analysis.

Specimen	Herbarium	No. on map (Fig. 2)
Kuban' region		
<i>Ex herbario Horti botanici Jurjevensis</i> . Headstreams of Kuban, Uchkulang. On rockslides. Very rare. VII-1900. Legit N. Desoulavy, det. W. Lipsky.	[MW!]	1
Daghestan		
Resp. Autonoma Daghestania. Districtus Akhty, p. Kurusch, ad fontes flumini Tschechitschaj. I/VII-1990. A.P. Khokhriakov.	[MW!, MHA!]	2
Resp. Autonoma Daghestania. Flumen Tscharyntsichaj. 2/VII-1990. A.P. Khokhriakov.	[MW!, MHA!]	3
Daghestan, Akhty distr., Kurush township, Mular mountain. h = 2800 above sea level. 2/VIII-1991. A.P. Khokhriakov.	[MHA!]	4
Daghestan, Nesen-Dagh mountain. 5/VII 1990. A.P. Khokhriakov.	[MHA!]	5
Tsumada distr., near meteorological station "Sulak highland". Southern slope, on rockslides, 3300 m. 14/08/1997. R.A. Murtazaliev.	[MW!]	6
Eastern Caucasus, Daghestan, headstreams of riv. Usukh-Chai (feeder of riv. Samur), Bazar-dyuzi mountain, to south from village Kurush, on slate rockslides, h. 3000 m. 24 July 1970. V. Prima.	[MW!]	7
Nakhichevan region		
Respublica autonoma Nachiczewan, in monte Kapudzhikh jugi Zangezurici, inter schisti fragmenta, ca. 3200-3300 m. s. m. Leg. A. Takhtadzhian et S. Czerepanov. 1950 VIII 14.	[MW!]	9
Turkey		
E. Bourgeau. - Plantae Lyciae. 1860. In petrosis regionis alpinae montis Ak-Dagh. S. Julio.	[MW!]	10
Iran		
<i>Hortus Botanicus "Ariamehr"</i> . Collector: H. Foroughi. Place: Nesa Karaj valley. Date: 13 May 1971. Alt.: 2110.	[MHA!]	11
<i>Hortus Botanicus "Ariamehr"</i> . Collector: M. Fotovat. Place: Kelardasht. Kuhetakhtesoleyman. Slope: E. Soil: clay. Locality: rocky. 12 July 1973. Alt.: 3620.	[MHA!]	12
Armenia		
Armenian SSR, Kotayk district, Gegham ridge, lake Akna-litch, rubble rockslides on southern slope, 3300 m above sea level. 28.08.1947. Leg. A. Akhverdov, det. I. Arevshatyan.	[ERE!]	13
Armenian SSR, Kotayk district, Agmagan mountain, lake Akna-litch, NE shore, black rockslide, southern slope. 04.08.1960. Leg. et det. E. Gabrielyan.	[ERE!]	14
Gegham ridge, near Akna-litch lake, NW slope of mountain Eratumber I, 3200 m above sea level. High ascent strongly rank slope. 08.1968. Leg. A. Akhverdov, N. Mirzoeva. Det. A. Akhverdov.	[ERE!]	15
Armenia, Syunik province, Sisian distr., vicinity of Ughtasar mountain, alpine meadow, south slopes, 3320 m, N 39°41'15" E46°03'14". 02 August 2007. Leg. I. Gabrielyan, A. Bruch. Det. I. Gabrielyan.	[ERE!]	16

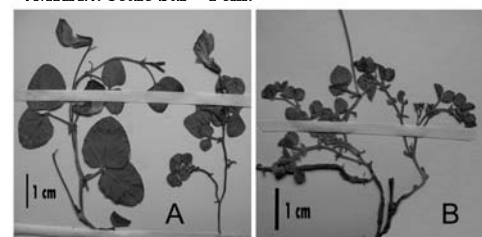
Results and Discussion

Morphological variations

When subdividing a single genus (regardless of *Vavilovia* Fed. or *Alophotropis* (Jaub. & Spach) Grossh.) into some intrageneric units, most authors agree that leaf features should be used as a key basis for division (4, 5). Two main distinguishing traits were cited, size and shape of the leaflet. According to A.A. Grossheim (5), plants from Southern Transcaucasia, Nakhichevan and Karabakh (*Alophotropis aucheri*, see Table 1) are characterized with smaller leaflets with keel-like basement (obovate shape). In contrast, plants from western and eastern Caucasus possess larger ovate leaflets with rounded basement (*A. formosa*) (Fig. 2).

Analysis of herbarium material available ensures that *Vavilovia*, similar to most legumes, exhibits expressed leaf anisophylly (Fig. 2). The first leaves of axillary sprouts bear leaves differing from leaves borne on upper nodes. For example, the first leaves on shoots of Daghestan plants possess tiny obovate leaflets while upper leaves are characterized with large ovate leaflets.

Figure 2. Herbarium specimens of *V. formosa* demonstrating a wide range of variation of leaf size and proportion. A: Daghestan, river Charyn-Chai, on rank rockslide, 2.07.1990. A.P. Khokriakov [MHA!]; both plants originate from the same population. B: Respublica autonoma Nachiczewan, in monte Kapudzhikh jugi Zangezurici, inter schisti fragmenta, ca. 3200-3300 m. s. m. Leg. A. Takhtadzhian et S. Czerepanov. 1950 VIII 14 [MHA!]. Scale bar = 1 cm.



All Armenian plants possess clearly obovate leaflets ($l_d/l_p < 1$) with expressed acro-basiscopic asymmetry (Fig. 3A). The t-test reveals significant differences between Armenian samples and all other samples ($p < 0.01$). Daghestan samples are characterized with large ovate (sometimes almost triangular, $l_d/l_p > 1$) leaflets. The specimens from the Nakhichevan' region are remarkable with their tiny rhomboid (sometimes scarcely roundish, Fig. 1) leaflets. These plants together with those from Turkey and Iran form a transient group ("N-I-T group" on Fig. 3) between Daghestan-Kuban' and Armenian groups (Fig. 3A). The Iranian plants are characterized with comparatively large oblong "lathyroid" leaflets (although certain anisophylly is observed). Notably, many leaflets express "normal" acro-basiscopic leaflet asymmetry ($h_a/h_b < 1$), i.e. the basiscopic part is wider than acroscopic which is typical for most legumes.

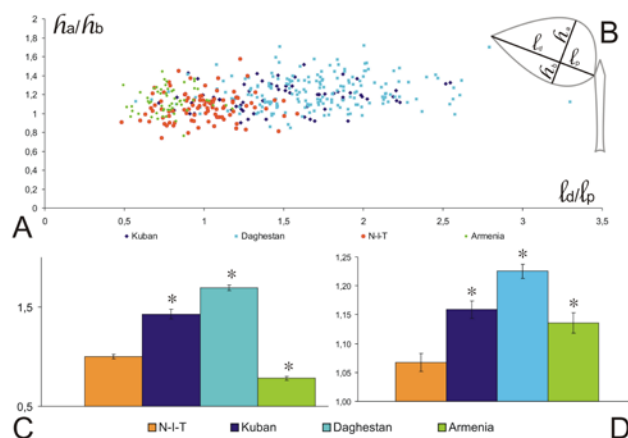
No distinct separation was observed for leaflet characters thus ensuring that leaf differences cannot be referred to as interspecific. Leaf traits seem quite variable in ontogeny and in different areas of the habitat. Possibly material from the same region collected on different elevations might enable research on the interrelation between leaf morphology and elevation where collections were made.

Earlier work (11) on DNA (RAPD) polymorphism reveal two supported subclades within a single *Vavilovia* clade, one of them being composed of Armenian and Nakhichevan samples and another of Daghestan and Kuban'. Samples from other regions of the habitat (e.g., Turkey) were not investigated in the presented molecular studies; therefore, no comparisons can be made.

It is problematic now to fully reconstruct the initial area of habitat of *Vavilovia*. To date, it inhabits few regions and seems to have no interchange of genetic information between them. This case represents the initial steps of allopatric speciation, and the first results of prolonged separation can be easily seen as morphological and DNA differences between populations along different sides of the Caucasus Mountains. One may propose that disjunction of *Vavilovia* populations occurred in stages: the initial area of habitat was first separated by a mountain ridge and then both halves split into other minor loci. The ancestral area of habitat may be connected with the Middle East, as all southern plants (Iran, Turkey and Nakhichevan) are significantly similar ($p < 0.01$) and differ from those from other regions. Kuban' specimens also exhibit proximity to the Daghestan samples according to molecular data (11); however, phenotypic differences between them are significant. The alternative scheme could include invasion of *Vavilovia* from ancient habitats (probably corresponding to southern part of contemporary area) throughout the Caucasus from the South to the North, expansion into new territory and then partial extinction in both primary and secondary areas. Regardless of the scenario chosen, one needs to assume that northern and southern populations of the Caucasus existed as separated entities for a long period, although morphological data (as compared to molecular ones) provide no estimation on longevity of such a separation. The most morphologically diverged group is located in Armenia.

The paucity of any arduous *Vavilovia* material leads to the fact that almost no data on intraspecific or intrageneric variability of this plant are available. Some reports on variability of flower color (pale pinkish or purple), pubescence (*P. formosum* var. *pubescens* C.C. Townsend (12)) and leaf morphology (see above) were cited. Some variation in anthocyanin pigmentation of vegetative parts was reported by R. Makasheva

Figure 3. Scatterplot distribution of quantitative traits of studied samples (A); scheme of *Vavilovia* leaflet with measurements indicated (B); meanings of l_d/l_p (C) and h_a/h_b ratios. Average meaning and SD is presented on C and D, asterisk indicate significant differences from the N-I-T (Nakhichevan - Iran - Daghestan) group.



et al. (6) and are clearly seen on fine photo images presented in (7). Flower and leaf pigmentation features are unfortunately lost in herbarium samples. However, in accordance with N.I. Vavilov's law of homologous series (13), much wider variability can be expected in *Vavilovia* populations since much is known on interspecific (including mutational) variations in its close relative, *Pisum* s. str. As no specific status was attributed to different morphological novelties in pea (authors of (10) interpreted them as varieties), the existing differences in leaflet morphology can hardly be interpreted as interspecific or distinguishing subspecies of *Vavilovia*. Additional samples and collections of this plant are of great interest, especially material from other parts of hypothetical habitats. For example, absence of accessible collections from the territory of Georgia is remarkable and may be evidence for inadequate exploration in *Vavilovia* habitats.

On the other hand, the most widespread tool in taxonomical interpretations is the subjective will of specialists interested in a certain taxon. To date, no special investigations on heritability of various traits in *Vavilovia* have been conducted. For example, no crosses between different forms were performed and no clear data on morphological variation between plants from different areas of the habitat (with special reference to ontogenetic and environmental plasticity which overlap leaf differences) exist. It seems more correct to avoid conclusions on the status of *Vavilovia* forms. They can hardly be interpreted as different species, but probably correspond to different varieties or even subspecies. The level of molecular polymorphism distinguishing them is at least comparable to that between different subspecies of *Pisum* s. str.

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An archaeobotanical and molecular fairy tale about the early Iron Age Balkan princess and the charred pea

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Pea (*Pisum sativum* L.) is one of the most important crops in Serbia and other Balkan countries. It is used for human consumption, animal feeding and for various non-food uses such as green manure (1). Apart from the cultivated pea (*P. sativum* subsp. *sativum*), the flora of the southeast regions of Serbia is rich in 'tall pea' (*Pisum sativum* subsp. *elatus* (Steven ex M. Bieb.) Asch. & Graebn.), most likely being one of the northernmost borders of its distribution in southeast Europe (2).

Pea is one of the most ancient and the most persistent grain legume crops in the archaeological findings of the Balkans, where the 'agricultural revolution' of Europe began about 9000 years ago. In Serbia, especially in its northern parts, pea is found in tells belonging to Neolithic times, such as the site Gomolava, Bronze Age, with Židovar and Feudvar, Early Iron Age, with Gomolava, Feudvar and Gradina-upon-Bosut, up to La Tène, represented by the tell of Gomolava (3). Together with lentil (*Lens culinaris* Medik.), chickpea (*Cicer arietinum* L.), bitter vetch (*Vicia ervilia* (L.) Willd.) and some other grain legume and cereal crops, pea travelled up the Danube valley into the continental interior and quickly reached its farthest regions (4).

Charred plant remains are possibly the most unexpected type of preserved biological material to have yielded ancient DNA (aDNA), and even though they are fragmented, they still contain information which may help us to understand agricultural or vegetation history (5, 6). Ancient DNA has received much attention since the mid-1980s when the first sequence of an extinct animal species was recovered from a museum specimen (5, 6). Since then, the majority of ancient DNA studies have focused predominantly on animal species, but the investigation of plant aDNA was limited, with the exception of cultivated species found in archaeological sites. There have been studies on charred archaeological samples of other species such as maize and wheat (7). However, there are still problems with charred plant ancient DNA - primarily the enhancement of DNA degradation upon charring. Despite these problems, investigation of ancient plant DNA may provide opportunities for palaeogenetic studies in plants for phylogenetic analysis and species identification (8).

Materials and Methods

The fortified hill settlement of Hissar in Leskovac is a multilevel settlement belonging to the Brnjica cultural group in the Morava Valley and dates back to 1350-1000 B.C. in the Iron Age I (9). At an altitude of 341 masl the Hissar hill has a strategic position over the confluence of Jablanica and Veternica rivers in South Morava and over the greatest part of the Valley of Leskovac. It is fifty kilometers long and forty-five kilometers wide.

The archaeological excavations at Hissar began in 1999, and in 2005 two rich grain legume samples were collected from the deposits belonging to Brnjica II a level from the 12th century B.C. Flotation of 7 liters of earth substrate yielded 2572 charred seeds of pea (Fig. 1). In one of the previous archaeobotanical reports (10) only one uncertain

Figure 1. Charred pea seeds from the Iron Age fort Hissar, southern Serbia



record of pea from the deposits of Brnjica cultural group was made. In addition to pea, a collection of bitter vetch with about 3000 seeds and supplements of faba bean and lentil was found.

The 50 g of charred pea seeds were ground to as close a powder form as possible in liquid nitrogen with a mortar and pestle. During grinding insoluble polyvinylpyrrolidone (PVPP) was added. The resulting powder was added to 1 ml CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, preincubated at 60 °C), mixed thoroughly and incubated at 60 °C overnight. The mixture was centrifuged in a microfuge for 10 min at 14,000 rpm, and the supernatant extracted twice with 24 : 1 (v/v) chloroform : isoamyl alcohol. Two volumes of CTAB precipitation buffer (1% CTAB, 50 mM Tris-HCl pH 8.0, 10 mM EDTA) were added to the aqueous layer and mixed well. The mixture was incubated at room temperature for 1h and then centrifuged for 10 min at 14,000 rpm. The supernatant was discarded and the pellet was suspended in 50 µl sterile distilled water. The concentration of DNA was measured using a NanoVue spectrophotometer (GE Heltcare, Little Shalfont, UK). For genomic amplification, the GenomiPhi DNA Amplification Kit (GE Heltcare, Little Shalfont, UK) was used according to manufacturer's instructions. The PCR reaction was performed using primers for 26S rRNA: 26Sf: 5'-attcccaacaacccgactc-3'; 26Sr: 5'-gccgtccgaattgtagctg-3'. The reaction mixture (25 µl): 1 µl DNA, 2.5 mM MgCl₂, 0.2 mM dNTP mix, 0.4µM primers, 1X Taq buffer with KCl (Fermentas) and 1U Taq polymerase (Fermentas). The PCR reaction was performed in a Biometra Thermalcycler, using the following profile: 95 °C for 5 min (initial denaturation); 30 cycles - 95 °C for 30 s (denaturing); 60 °C 30 s (annealing); 72 °C 1 min (extension); with a final extension for 10 min at 72 °C. For positive and negative control reactions genomic DNA from pea and water were used, respectively. The PCR products were separated on 2% agarose gel.

Results and Discussion

It is almost impossible to precisely determine a charred pea seed solely by its morphology if it misses its specific coffee-bean-shaped hilum (Fig. 2). The seed shape, regularly spherical with few flattenings depending on their position and space within a pod, and the seed size are not sufficient to determine if one deals exactly with pea. The reason for this lies in the fact that pea seeds can be as small as some *Vicia* seeds and, if the hilum is lost, they cannot be differentiated from each other.

In the Hissar pea sample, only few seeds maintained an almost intact testa with hilum. Fortunately, among the pea seeds, 32 seeds of lentil, bitter vetch and faba bean were observed and their identity was quite clearly determined (Table 1). The seeds of lentil and bitter vetch differed from pea in shape and from faba bean in size. By applying the exclusion principle, it is almost certain that the remaining seed were pea.

Figure 2. A charred pea seed from Hissar with preserved important morphological features (drawing by A. Medović)

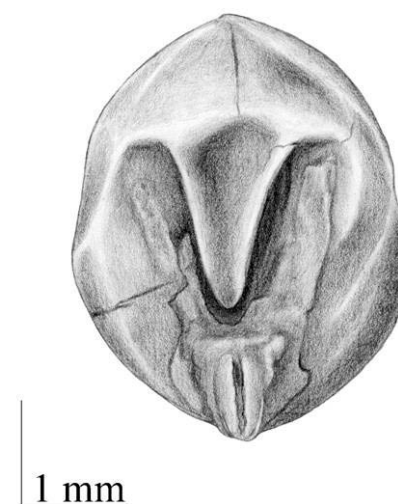


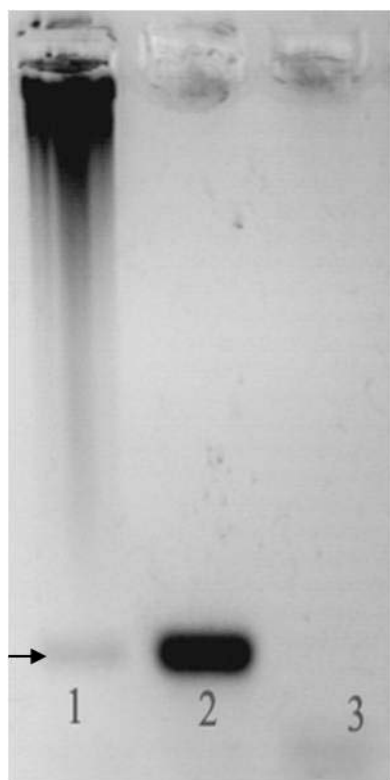
Table 1. The specification of the Hissar pea sample.

Remain type	Quantity (number)	Quantity (%)	Mass (mg)	Mass (%)
Pulses				
<i>Pisum sativum</i>	2572	85.68	62768	97.82
<i>Lens culinaris</i>	21	+	113	r
<i>Vicia faba</i>	8	r	472	+
<i>Vicia ervilia</i>	3	r	21	r
Cereals				
<i>Hordeum vulgare vulgare</i>	10	r	126	r
cf. <i>Triticum aestivum</i> s.l.	1	r	6	r
<i>T. aestivum</i> s.l., rachis internodes	1	r	3	r
<i>Triticum dicoccon</i>	1	r	11	r
<i>Triticum monococcum</i>	1	r	2	r
Cerealia indeterminata	1	r	8	r
Millets				
<i>Panicum miliaceum</i>	320	10.66	553	+
Oil and fibre plants				
<i>Linum usitatissimum</i>	21	+	20	r
cf. <i>Lallemantia iberica</i>	1	r	2	r
Dye plants				
cf. <i>Carthamus tinctorius</i>	1	r	2	r
Fruits				
<i>Sambucus</i>	1	r	0	r
Weeds and ruderals				
<i>Convolvulus arvensis</i>	16	+	40	r
<i>Echinochloa crus-galli</i>	8	r	2	r
<i>Polygonum convolvulus</i>	4	r	5	r
<i>Chenopodium album</i>	3	r	0	r
<i>Setaria viridis</i>	2	r	0	r
<i>Digitaria sanguinalis</i>	1	r	0	r
<i>Galium aparine</i>	1	r	2	r
<i>Lolium temulentum</i>	1	r	3	r
<i>Medicago</i>	1	r	2	r
Plant families				
<i>Fabaceae</i>	1	r	3	r
<i>Poaceae</i>	1	r	0	r
Total	3002	100	64164	100
Other finds				
Insecta, larva	1		0	

r < 0.5%; 0.5% < + < 1.0%

Since this work was also aimed at investigating the possibility of applying molecular methods to analyze archaeological pea remains, the DNA from ancient pea seeds was isolated and fragments of 26S rDNA were amplified by PCR. The standard DNA extraction procedure without genome amplification did not provide adequate quantities of DNA material for the PCR reaction. The DNA concentration in the isolate was 4.5 ng μl^{-1} and total yield of DNA was about 200 ng from 200 mg of seeds. However, using the GenomiPhi DNA Amplification Kit for random amplification of genomic DNA and using the product of this reaction in PCR we observed a single band (150 bp) corresponding to the gene for 26S rRNA (Fig.3).

Figure 3.
Electrophoretic separation of the PCR products of the charred pea seeds from Hissar: (1) the PCR product indicated by the arrow and obtained with ancient pea DNA, (2) positive control and (3) negative control.



Conclusions

Despite the fact that pea was one of the first cultivated crops, discovery of 2572 pea seeds in a single sample at Hissar represents a unique example in the archaeobotany of southeast Europe. The pea seeds are often poorly represented in the archaeobotanical records, not only due to rather small chances of being carbonized, but also because of the difficulties related to their proper determination. Pea at Hissar was a crop of its own and was stored separately from other crops. It also seems that pea was cultivated on small, patch-like fields where intense crop care was implemented.

These results encourage future research to use standard DNA extraction procedures, with the addition of insoluble PVPP and an extended period of incubation, accompanied by random genome amplification to obtain appropriate material for molecular analyses of archaeological plant material. The charred pea seeds contained aDNA, which could be used for further molecular analyses.

Acknowledgements

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Words denoting pea (*Pisum sativum*) in constructed languages

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Introduction and Aim

It is no wonder that the words denoting pea (*Pisum sativum* L.), one of the most ancient crops, are present in both extinct and living languages of the Old World, especially in Europe, Asia Minor, Near East and Central and northeast Asia. In many cases, the words denoting pea retained their original form and meaning, despite a millennia-long period between proto-languages and their modern descendants (1).

The term *constructed language* refers to the one not evolving naturally but being consciously invented for a specific purpose. The most numerous among these are auxiliary languages, made with a primary goal of enhancing the communication between people not sharing a common native language. Their development reached its peak in the mid-20th century with Esperanto. In addition to auxiliary languages, there are other sorts of constructed languages. Among them are artistic and alternative languages. The former comprise fictional languages that are used in literature while the latter represent languages that could have existed if historical events had been different. This research was aimed at collecting the words denoting pea in major constructed languages.

Materials and Methods

The words denoting pea were collected from all available printed and electronic dictionaries of major constructed languages and other related relevant resources. The origin of these words was investigated and assessed after they were grouped.

Results, Discussion and Conclusions

The words denoting pea in a large majority of the auxiliary languages (Table 1), such as the Esperanto *pizo* or the Volapük *pisäl*, are based upon the Latin *pisum*, retaining its meaning. Some auxiliary languages such as Modern Indo-European or Slovianski aim at developing languages that would closely resemble one of the real proto-languages, in their case Proto-Indo-European and Proto-Slavic: by that reason, the words denoting pea in these two, *kikēr* and *groh*, closely resemble the Proto-Indo-European **kek-* and the Proto-Slavic **gorxŭ*.

Table 1. Words denoting pea in some constructed languages.

Division	Genre	Language	Word	Division	Language	Word
Artistic	Alternative	Brithenig	<i>pis</i>	Auxiliary	Interlingua	<i>piso</i>
		Novegradian	<i>grášina</i>		Lingua Franca Nova	<i>pi</i>
		Wenedyk	<i>pies</i>		Modern Indo-European	<i>kikēr</i>
	Fictional	Quenya	<i>orivaine</i>		Novial	<i>pise</i>
Auxiliary		Esperanto	<i>pizo</i>		Occidental (Interlingue)	<i>pise</i>
		Glosa	<i>pisum</i>		Slovianski	<i>groh</i>
		Idiom Neutral	<i>pis</i>		Universalglot	<i>piso</i>
		Ido	<i>pizo</i>		Volapük	<i>pisäl</i>

The alternative artistic languages such as Brithenig and Wenedyk represent a scientifically based construction of two hypothetical Romance languages, where Latin influenced Insular Celtic in the former and West Slavic influenced the latter. Therefore, the Brithenig *pis* is similar to the real Welsh

pysen, while the Wenedyk *pies* is a hypothetical Polish-like derivation of the Latin *pisum*. Novegradian is a supposed North Slavic language, with *grášina* as a local term denoting pea. The word denoting pea in Quenya, an Elvish language invented by J. R. R. Tolkien, is *orivaine* (2), consisting of *orë* (*seed* or *grain*) and *vaine* (*sheath* or *pod*).

The words denoting pea in the most widely spoken constructed languages, together with very few other crops, simply witness the significance of pea in our everyday lives.

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Mapping the *st* locus in respect to three molecular markers on Linkage Group III

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The overwhelming majority of genetic studies in recent years have been based on DNA markers. Polymerase chain reaction (PCR)-based DNA markers have been recognized to be powerful tools for rapid construction of genetic maps. However, morphological markers still remain very convenient due to their ease of scoring and allowing larger plant numbers to be analyzed.

A pea consensus linkage map was published in 1998 (1) which included mainly morphological and isozyme data known to date. Integration of new molecular, biochemical and morphological data (2) into existing maps has been possible through the use of a number of anchor genes (3). The *st* gene on Linkage Group III is a convenient genetic marker manifested as reduced stipules (4) that is clearly seen in young seedlings. The recommended anchor markers in LGIII are *Aat-c*, *Adh-1*, *M*, *st*, *b*, *Lap-1* (3). In the present work, we mapped the *st* locus with respect to the morphological marker *b* and three molecular markers based on the following DNA sequences available from public databases: AF255058 *Pisum sativum* 33 kDa ribonucleoprotein gene, complete cds; AF280748 *Pisum sativum* phospholipase C gene; and AJ832139 *Pisum sativum* sym7 gene for GRAS family protein.

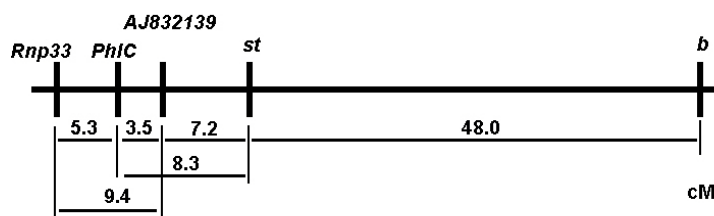
The latter gene is defined in the database as "sym7" (5); however, it seems to be different from the marker with the same designation "sym7" described by (6) and mapped on LGIII distally to *b* (1). To avoid confusion we designate it here as AJ832139, while the former two markers are designated as *Rnp33* and *PhlC* as in (7, 8). The molecular markers used were assessed with the use of CAPS (9) and the following primers and restriction enzymes were used:

Primers used	Primer sequence	Restriction enzyme	Length of PCR-product (bp)	Fragment lengths in WL1072 (bp)	Fragment lengths in VIR320 (bp)
Rnp33_1f Rnp33_2r	5'ATGTCGTGTAAGTCCACCACT 5'ctgtcttcagcaacacttact	<i>Tru9I</i>	2034	622 296 a number of smaller fragments	approx. 400 approx. 200 a number of smaller fragments
PhlC_1f PhlC_9r	5'CACAGAGAATGAAGCACAATC 5'ccttcaagctttccgagcta	<i>HpaII</i>	963	694 269	351 343 269
AJ832139_14f AJ832139_13r	5'GCGACTTAGCTCGAGTGATA 5'tttgcaagcttaccggaac	<i>BstFNI</i>	958	551 407	551 206 201

One hundred twenty-two plants of an F₂ population from the cross, WL1072/VIR320, where WL1072 carried *st* (reduced stipules) and *b* (pink flowers), were analyzed for the genotype with respect to *Rnp33*, *PhlC* and AJ832139, as judged on fragment lengths formed after endonuclease treatment of PCR-products and characterized for *st* and *b*. Approximately 10 seeds collected from *St*- plants were sown to assess the allelic state of the *st* locus. The F₂ progenitors of the F₃ families segregating for normal/reduced stipules

clearly seen in young seedlings were regarded as heterozygous. Mapmaker 3.0 software was used to construct the genetic map of the LGIII segment in Figure 1.

Figure 1. Fragment of the linkage map of LGIII.



In this study we mapped three molecular markers closely linked to *st*. One marker, AJ832139, had not been previously mapped. The choice of *st* was due to its ease in scoring and convenient use in mapping experiments related to the central region of LGIII. The *b* locus appears to be quite distant to *st* in our population.

Acknowledgement

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Yellow green (*yg1*) mutation in *Pisum*

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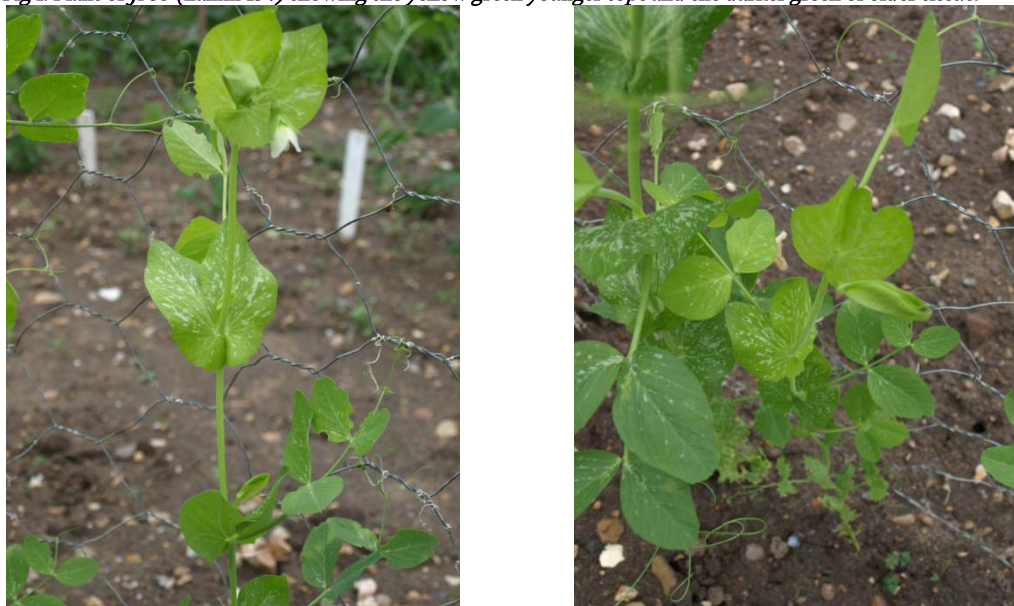
John Innes Centre, Norwich, UK

The yellow green mutant was first described by Kellenbarger in 1953 (1). The mutation arose as a single plant (KE_{sl}) in a crop of Alaska. Seedlings bearing the mutation were reported as being of uniform yellowish green when they emerge with growth becoming a normal green over time. In 1953 Lamprecht notes the work of Kellenberger and reassigns the mutation as *vil* (2). Blixt, in his 1971 thesis on mutations in *Pisum* (3), designated the mutation as *chlorina-virescens* and lists it under *vil* but goes on to comment that the question of reinstating the *yg* symbol should be discussed. While Blixt was aware of the mutant the line does not appear in the Weibullsholm collection and the mutation is logged as extinct in the gene database at the time of writing (4).

As part of the regular regeneration of germplasm against wire in the field, the abnormally pale foliage colour of one particular accession was noticable. The tops of the plants were all yellowish green becoming a mid green lower down the plant. Documentation associated with this line showed it was received at the John Innes in 1964 from Robert Lamm. A letter from Lamm that accompanied the material states that this line originated from Kellenbarger and included alongside the symbols *yg1* and *vir1*.

The chlorophyll content of leaf tissue was measured using a portable Minolta SPAD 502 chlorophyll meter which 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. Readings from the leaflets and stipules from the top of the plant gave readings of 18.5 SPAD units which doubled to 34.0 SPAD units seven nodes further down the plant. While the reading obtained from foliar tissue lower down the plant is in the mid range of values measured in other pea germplasm, the value of the younger foliage is at the low end (5).

Fig 1. Plant of JI 35 (Lamm 134) showing the yellow green younger tops and the darker green of older tissue.



It should be noted that the line differed from the founder line Alaska in one further respect in having reduced wax on the stipules. This was not noted by Lamm in the accompanying notes associated with the line. The genetic relationship between the yellow green foliage and reduced stipule wax awaits further study.

As the line has not been studied or published on for such a long period of time, I propose to reinstate the original gene symbol *yg* as valid and *vil* as a synonym in the PGene database (4). The status of the mutation moves from extinct to valid and seed is now available from the John Innes Pisum Collection.

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Genistein and methyl jasmonate inhibit nodulation of *Pisum sativum*

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Low root zone temperatures (RZT) early in the season can lower the nodulation rate of rhizobium bacteria on peas (*Pisum sativum* L.), lowering their ability to fix nitrogen. Poustini et al. (1) found that treating *Rhizobium leguminosarum* bv. *phaseoli* with either genistein or methyl jasmonate can mitigate the effects of low RZT within beans (*Phaseolus vulgaris*). We wanted to know if these chemicals would also mitigate low RZT effects on peas, specifically testing two varieties, 'Mozart' and 'Majoret'. Our results suggest that these two chemicals actually inhibit nodulation rather than stimulating it in these varieties.

The experiment consisted of exposing *R. leguminosarum* bv. *pisi* cultures to genistein, methyl jasmonate, or both and inoculating imbibed pea seeds or pea seedlings with the cultures. We used a randomized complete block design consisting of 200 samples divided into four repetitions, two of which used vermiculite as a growing medium and two using Montana State University (MSU) potting soil. In addition, non-nodulating checks were included with each soil/cultivar combination that was inoculated with untreated rhizobium. The checks consisted of a non-nodulating mutant *sym14* in a 'Sparkle' background.

The rhizobium inoculant was prepared three days in advance of the inoculation date. Single colonies of *R. leguminosarum* bv. *pisi* were isolated from a commercial granular rhizobium source (NitraStik C, EMD Crop Bioscience), transferred to liquid media (2.2 mM K₂HPO₄, 1.8 mM NaCl, 0.8 mM MgSO₄, 1 gm/L yeast extract, pH 7.0) and shaken for 48 hours. Individual flasks of rhizobia were induced with specific treatments (genistein, 50 uM, methyl jasmonate, 20 uM) and shaken for 24 hours more. The peas were planted in conetainers and grown in a growth chamber set at 22°C during the 12 hr light phase and 16°C during the 12 hrs of dark. The seeds were initially inoculated 6 days after planting. Watering stopped two days before inoculation and began again two days later. A second inoculation was performed 4 weeks after planting, when the seeds had sprouted and the root system had formed. Harvest took place 2 weeks after the second inoculation, and the individual nodules on the roots were scored.

Nodulation rates in the various treatments are shown in Table 1. Nodulation on the control plants was relatively sparse, but it was four- to eight-fold greater than that observed in the samples treated with genistein. Samples treated with methyl jasmonate or with both chemicals displayed a complete lack of nodulation, as did the non-nodulating checks. Roots appeared healthy and vigorous under all treatments. The clear, negative impact of the treatments on nodulation rate discouraged us from pursuing similar experiments at other temperatures or with other varieties. We conclude that *R. leguminosarum* bv. *pisi* does not respond to genistein or methyl jasmonate in the same way as *R. leguminosarum* bv. *phaseoli* and that treatment with these chemicals will not aid in the establishment of a robust rhizobium/pea symbiosis under cold field conditions.

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Table 1. Nodulation rate observed on roots under different treatments.

Cultivar	Treatment	Nodules per plant
Mozart	Distilled water	0.0
	Rhizobium only	2.1
	Rhizobium plus genistein	0.5
	Rhizobium plus methyl jasmonate	0.0
	Rhizobium plus both	0.0
Majoret	Distilled water	0.0
	Rhizobium only	1.2
	Rhizobium plus genistein	0.2
	Rhizobium plus methyl jasmonate	0.0
	Rhizobium plus both	0.0

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