PERIODICUM BIOLOGORUM
VOL. 112, No 3, 263–272, 2010

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Original scientific paper

Comparative seed morphology of *Trifolium* L. Species (Fabaceae)

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Key words: clover, micromorphology, seed coat, SEM, taxonomy, testa ornamentation

Abstract

Background and Purpose: *Seed morphological characteristics proved to be useful taxonomic features, helpful in identification of large number of species and genera. Seed characters, such as shape, size and seed-coat surface, have low phenotypic plasticity and are less affected by environmental conditions. The purpose of this study was to describe and compare external seed morphological characteristics of 38* Trifolium *species, and to evaluate their possible use for taxonomic considerations.*

Material and Methods: *The seeds were gained from genebanks and seed collections and examined using light and scanning electron microscopy (SEM). Obtained data were statistically processed using analysis of variance, Principal Component Analysis and Multiple Correspondence Analysis.*

Results and Conclusions: *The results showed that seeds were laterally compressed and bilobed and showed morphological variations in size, shape, color, seed coat ornamentation and morphology of the radicle lobe. Examined characters did not provide considerable information that could be used to distinguish subgenera or sections of this genus, because seed characteristics did not support the subgeneric classification. A unique seed coat morphology and combination of other seed characteristics permitted easy identification of some of the examined species, like* T. subterraneum, T. alexandrinum, T. angulatum *and* T. vesiculosum.

Received May 9, 2008.

INTRODUCTION

Seed morphology and the structure and morphology of the seed coat has been found to be a useful taxonomic feature *(1, 2, 3, 4)*. Seed characters are very helpful for identification of a large number of species or genera *(5, 6, 7, 2)*. In many cases, morphological characteristics, such as seed shape and testa ornamentation, can be used to distinguish species and varieties *(8)*.

Fruits and seeds tend to show less phenotypic plasticity, in comparison with other organs *(9)*. Seed characters are less affected by environmental conditions, and often reflect genetic differences. Remarkable variability in seed morphology exists in Angiosperm taxa, with relative constancy of seed structure in narrow taxonomic units *(4, 10)*. According to Esau *(10)* in external topography the important features of the seeds are shape, size, seed-coat surface, placement of the hilum and presence of different structures, such as aril, caruncle or elaiosoms.

Preliminary studies of the seed coat surface of 24 *Trifolium* species were made by Zohary and Heller *(11)*. They found that the surface of the seed was smooth, roughened, tuberculate, wrinkled or pitted and

described five types of seed coat patterns. The seeds of this genus vary in their dimensions, weight and shape. Algan and Büyükkartal *(1)* described ultrastructure of the seed-coat of *Trifolium pratense*. According to their findings, the outer layer of the seed coat is composed of elongated macrosclereids. Under this layer, a hypodermal layer occurs, made up of I-shaped osteosclereids (hour-glass cells). Beneath these layers, the integument consists of parenchyma cells. Slattery *et al. (12)* analyzed the color of the seed coat of *T. subterraneum* and its relationship with phenolic content and impermeability. They concluded that the darkening of the testa was associated with oxidation of phenol by catechol oxidase. Taia *(13)* examined seeds of *Ononis, Melilotus, Trigonella, Medicago* and *Trifolium,* in order to assess the relationship between them. The obtained results supported the separation of *Trifolium* and *Ononis* in separate subtribes. The author reported oval and global shape of *Trifolium* seeds and smooth, striate and wrinkled seed coat pattern. None of this studies illustrated completely the ranges of variability in seed morphological and micromorphological characters of *Trifolium* species. Several species of this genus are cultivated as economically important fodder crops. Seeds of such plants are often used and transported with no collateral plant parts to aid in their identification, so it is particularly important to obtain better knowledge of their morphological characteristics, which could help in determination of species *(14)*.

The purpose of this study was to describe and compare external seed morphological characteristics of 38 *Trifolium* species, and to evaluate their possible use for taxonomic considerations.

MATERIAL AND METHODS

Seeds of 38 *Trifolium* species used in this study were gained from genebanks and seed collections which are listed in Table 1. Infrageneric classification was given according to Zohary and Heller *(11)*. Dry, mature seeds were observed under scanning electron microscope (SEM). The seeds were mounted on stubs using double-sided adhesive tape, sputter coated with gold for 180 seconds, 30 mA (BAL-TEC SCD 005) and viewed with JEOL JSM-6460LV electron microscope at an acceleration voltage of 20 kV. For light microscopy observations and measurements, the seeds were observed using Image Analyzing System Motic 2000. The observations were made on thirty randomly selected seeds of each species. The detailed structure of the seed coat was observed far from the hilum. Terminology followed Stearn *(15),* with some modifications. Weight was obtained for 100 seeds, in five replicates, using an electronic balance, and, after recalculation, presented as the weight of 1000 seeds. The seed index was calculated as the ratio of the seed length and the seed width. Data were statistically processed using Statistica for Windows 7.0. *(16)*. Means, standard errors, correlations and coefficients of variation were calculated. Significance of differences were determined using Duncan test. The general structure of sample variability was established by principal component analysis (PCA), us-

TABLE 1

Voucher data for specimens used in the SEM studies.

Species	Origin	Code number
T. alexandrinum L.	Tunis	TRIF 275/02 (c)*
T. alpestre L.	USSR	59-S-TI-4; 314116 (a)
T. alpinum L.	France	1113 (b)
T. angulatum Waldst. & Kit.	USSR	81-S-230-3; 618308 (a)
T. angustifolium L.	Turkey	11-S1-16; 177562 (a)
T. arvense L.	Spain	49-S-6-4; 244322 (a)
T. aureum Pollich	France	1115 (b)
T. badium Schreber in Sturm	France	1116 (b)
T. campestre Schreber in Sturm	Greece	S-8-22; 260641 (a)
T. dalmaticum Vis.	England	S-225-1; 516292 (a)
T. diffusum Ehrh.	Turkey	59-S-46; 204517 (a)
T. dubium Sibth.	USA	S-13-4; 291782 (a)
T. fragiferum L.	USA	S-16-30; 239963 (a)
T. hybridum L.	Turkey	S-19-6; 204923 (a)
T. incarnatum L.	Morocco	S-20-12; 418900 (a)
T. medium L.	USA	99-S-25; 206485 (a)
T. micranthum Viv.	USA	41-S-195-1; 516359 (a)
T. montanum L.	Turkey	S-92-5; 205314 (a)
T. nigrescens Viv.	USA	S-28-1; 516371 (a)
T. ornithopodioides L.	England	51-S-188-2; 516374 (a)
T. pallescens Schreb.	France	1119 (b)
T. pannonicum Jacq.	Romania	S-30-8; 516377 (a)
T. patens Schreber in Sturm	France	29-S-67-2; 516386 (a)
T. pratense L.	USA	59-L38-96 (a)
T. purpureum Loisel.	Israel	S-76-5; 306627 (a)
T. repens L.	Morocco	S-35-7; 202518 (a)
T. resupinatum L.	India	S-36-85; 163314 (a)
T. retusum L.	Australia	S-208-1; 516424 (a)
T. rubens L.	USSR	51-S-72-4; 314123 (a)
T. spadiceum L.	Romania	S-105-3; 516434 (a)
T. stellatum L.	USA	41-S-43-6; 2332 (a)
T. striatum L.	USA	41-S-44-7; 516442 (a)
T. strictum L.	Portugal	09-S-78-1; 238372 (a)
T. subterraneum L.	USA	89-S-47-19; 516444 (a)
T. thalii Vill.	France	1121 (b)
T. trichopterum Pancic	Turkey	91-S-234-6; 120145 (a)
T. velenovskyi Vandas	Bulgaria	71-S-241-1; 90-139 (a)
T. vesiculosum Savi	Italy	S-90-7; 233816 (a)

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ing a standardized basic matrix. Multiple Correspondence Analysis (MCA) was done in order to check the hypothesis that the analyzed sample was composed of groups which were differentiated from each other.

RESULTS

Based on light and scanning electron microscopy observations, the examined *Trifolium* species showed variation in qualitative and quantitative seed characteristics.

Seed size

Trifolium seeds are very variable in size (Tables 2, 3). According to the values of the seed length and width, the seeds are classified into four groups and coded as follows: 1 (*L*: 0.90–1.25mm; *W*: 0.70–1.00mm), 2 (*L*: 1.26–1.60mm, *W*: 1.01–1.30mm), 3 (*L*: 1.61–1.95mm, *W*: 1.31–1.60mm) and 4 ($L>1.96$mm, $W>1.61$mm). Those two characters are strongly positively correlated (0.77, $p<0.05$). The weight of 1000 seeds varies from 0.20–0.80 g (1), 0.81–1.40g (2), 1.41–2.00g (3) and > 2.00g (4). It is also strongly positively correlated with seed length (0.88) and width (0.83). Species of the sect. *Paramesus* and *Chronosemium* are characterized by significantly the smallest seeds, compared to other sections (Table 2, 4). Significantly the largest seeds are those of sect. *Trichocephalum* and *Trifolium* (where much higher variability among the samples is obvious).

Seed shape

Trifolium seeds are laterally compressed and bilobed. The larger lobe is the lobe of the cotyledons, while the smaller is the lobe of the radicle. Hilum separates these two lobes and thus mostly determines the shape of the entire seed. They are usually round, ovoid, elongated-ovoid or heart-shaped (Table 4, Figures 1–6). Radical lobe can be prominent, with well defined groove between the lobes, or completely tightened to the other lobe, with

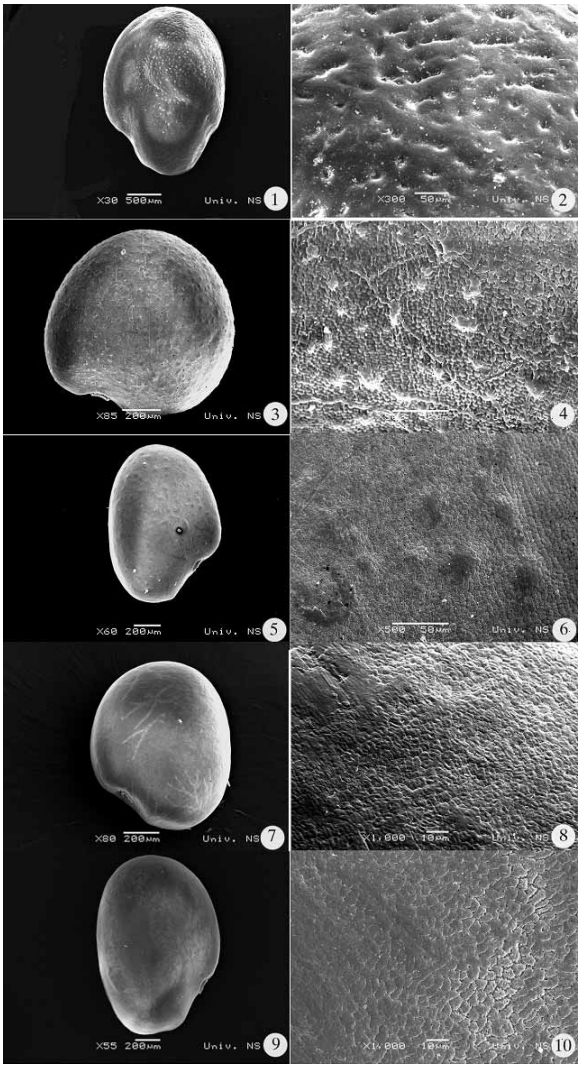

Figure 1. *Scanning electron micrographs of* Trifolium *seeds and details of seed coat surface. 1-2.* T. alexandrinum. *3-4.* T. angulatum. *5-6.* T. aureum. *7-8.* T. arvense. *9-10.* T. badium.

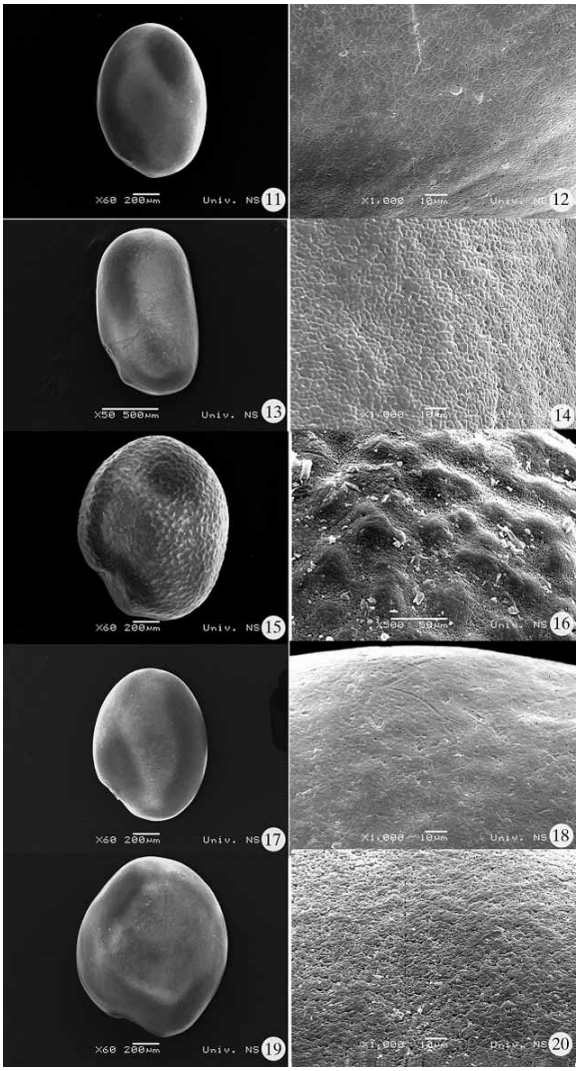

Figure 2. *Scanning electron micrographs of* Trifolium *seeds and details of seed coat surface. 11-12.* T. campestre. *13-14.* T. dalmaticum. *15-16.* T. diffusum. *17-18.* T. dubium. *19-20.* T. fragiferum.

TABLE 2

Quantitative seed characters of species from sections Lotoidea, Paramesus, Mistyllus, Vesicastrum and Chronosemium (means ± standard error, coefficient of variation %).

Section	Species	seed length (µm)	seed width (µm)	seed index	weight of 1000 seeds (g)
Lotoidea	*T. alpinum*	2418.4±78.3 (10.2)	2013.2±50.9 (8.0)	1.2±0.02 (6.5)	4.7±0.4 (11.3)
	T. angulatum	1008.4±30.9 (9.7)	801.2±17.1 (6.8)	1.3±0.03 (6.5)	0.4±0.03 (8.8)
	T. hybridum	1155.8±24.2 (6.6)	983.0±20.9 (6.7)	1.2±0.02 (4.7)	0.62±0.07 (18.2)
	T. montanum	1848.7±28.0 (4.8)	1317.2±36.2 (8.7)	1.4±0.04 (8.7)	1.6±0.4 (20.9)
	T. nigrescens	975.9±18.7 (6.1)	738.3±18.1 (7.8)	1.3±0.05 (12.2)	0.26±0.0 (1.0)
	T. ornithopodioides	1186.3±27.1 (7.2)	887.2±19.3 (6.9)	1.3±0.05 (11.0)	0.57±0.06 (17.0)
	T. pallescens	1499.4±25.3 (5.3)	1214.5±19.2 (5.0)	1.2±0.03 (7.9)	0.9±0.02 (3.1)
	T. repens	1064.1±32.5 (9.7)	1029.7±26.0 (8.0)	1.0±0.04 (11.1)	0.59±0.05 (13.7)
	T. retusum	937.6±19.1 (6.4)	809.0±23.3 (9.1)	1.2±0.03 (7.0)	0.33±0.01 (2.1)
	T. thalii	1296.1±27.6 (6.7)	1074.3±27.0 (8.0)	1.2±0.02 (5.3)	0.83±0.05 (7.7)
	average	1339.1±46.0 c (34.4)	1086.8±36.7 bcd (33.7)	1.2±0.01 ab (11.8)	1.04±0.3 bc (48.8)
Paramesus	*T. strictum*	1120.2±16.6 c (4.7)	889.6±16.4 cd (5.8)	1.3±0.01 ab (3.2)	0.48±0.0 c (0.0)
Mistyllus	*T. vesiculosum*	1479.4±27.2 bc (5.8)	1340.8±24.8 bc (5.8)	1.1±0.03 b (8.5)	1.2±0.02 bc (2.5)
Vesicastrum	*T. fragiferum*	1332.8±29.3 (6.9)	1121.3±22.43 (6.3)	1.2±0.02 (5.6)	0.98±0.2 (20.5)
	T. resupinatum	1470.5±42.7 (9.2)	1164.1±33.3 (9.0)	1.3±0.03 (6.6)	1.1±0.01 (1.4)
	average	1401.6±29.7 bc (9.4)	1142.7±20.1 bcd (7.9)	1.2±0.02 ab (7.1)	1.0±0.1 bc (22.6)
Chronosemium	*T. aureum*	1089.8±12.8 (3.7)	822.0±8.2 (3.1)	1.3±0.02 (4.1)	0.4±0.0 (1.5)
	T. badium	1528.6±42.4 (8.8)	1109.7±24.1 (6.9)	1.4±0.02 (4.4)	0.4±0.01 (3.5)
	T. campestre	1177.8±27.8 (7.5)	837.2±14.4 (5.4)	1.4±0.02 (4.1)	0.4±0.1 (14.6)
	T. dubium	1129.5±20.9 (5.8)	829.9±23.7 (9.0)	1.3±0.04 (9.8)	0.4±0.01 (1.8)
	T. micranthum	963.8±17.4 (1.8)	731.4±16.4 (2.2)	1.3±0.02 (4.2)	0.29±0.0 (0.0)
	T. patens	1249.5±19.3 (4.9)	783.6±12.2 (4.9)	1.6±0.02 (4.6)	0.4±0.01 (2.8)
	T. spadiceum	1247.6±21.6 (5.5)	833.6±17.0 (6.4)	1.5±0.02 (4.3)	0.5±0.01 (1.6)
	T. velenovskyi	1103.6±19.7 (5.6)	876.7±27.7 (10.0)	1.2±0.04 (9.6)	0.5±0.01 (2.8)
	average	1186.3±19.3 c (14.6)	853.0±13.4 d (14.1)	1.4±0.01 a (9.4)	0.41±0.02 c (19.9)

a shallow groove between them. According to the length of the radicle lobe, seeds are classified into three groups. The seeds of the first group have a very short radicle lobe, up to one half of the cotyledon lobe length (1). The species that belong to this group are *T. montanum, T. spadiceum, T. alpestre, T. medium, T. pannonicum* and *T. pratense*. The second group (2) is formed of species with radicle lobe of up to 2/3 of the cotyledon lobe length. This group is the largest and joins 25 of the examined species. All of the species from sect. *Chronosemium*, except *T. spadiceum*, belong to this group. The third group (3) is composed of the species with radicle lobe which is almost equal in length to that of cotyledons. Some species of sect. *Lotoidea,* sect. *Mistyllus* and *Vesicastrum,* as well as *T. trichopterum* from sect. *Trifolium* belong to this group.

Seed index values also determine the seed shape, and those two characters are significantly correlated (0.82, $p<0.05$). Their range is from 1.00–1.19 (1), 1.20–1.39 (2) and over 1.40 (3) (Tables 2, 3, 4). The highest values of seed index have seeds of elongated-ovoid shape, like *T. dalmaticum* (1.8), *T. patens* and *T. incarnatum* (1.6) (Figures 2, 3). The smallest values are recorded for *T. repens* (1.0), *T. vesiculosum* and *T. medium* (1.1) (Figures 3, 4, 6). Although small values are recorded for *T. medium*, *T. vesiculosum*, *T. retusum*, and *T. hybridum*, those seeds have very prominent radicle lobe, which makes them more heart-shaped (Figures 3, 5, 6). Among the examined sections, the largest values of seed index are recorded in sect. *Chronosemium*, while the smallest in sect. *Mistyllus*.

Seed color

The color of *Trifolium* seeds is very variable (Table 4). The seeds are purple, like in *T. subterraneum*, or their color varies from yellow to brown, yellow to green or light to dark brown. The color of the seeds is not correlated with other examined characters. Plants from sect. *Lotoidea* and *Trifolium* mostly have yellow to brown seeds.

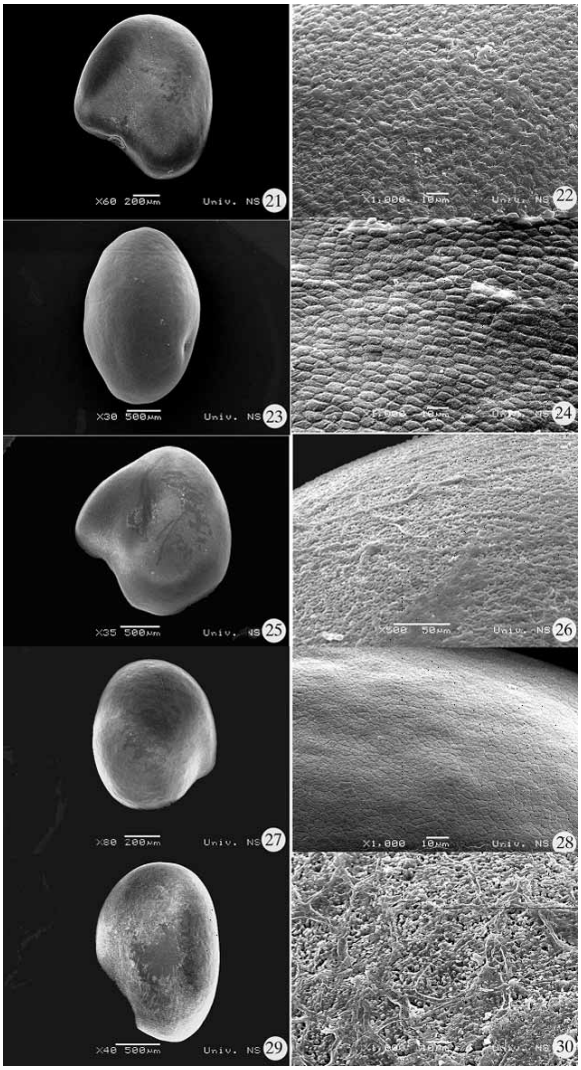

Figure 3. *Scanning electron micrographs of* Trifolium *seeds and details of seed coat surface. 21-22.* T. hybridum. *23-24.* T. incarnatum. *25-26.* T. medium. *27-28.* T. micranthum. *29-30.* T. montanum.

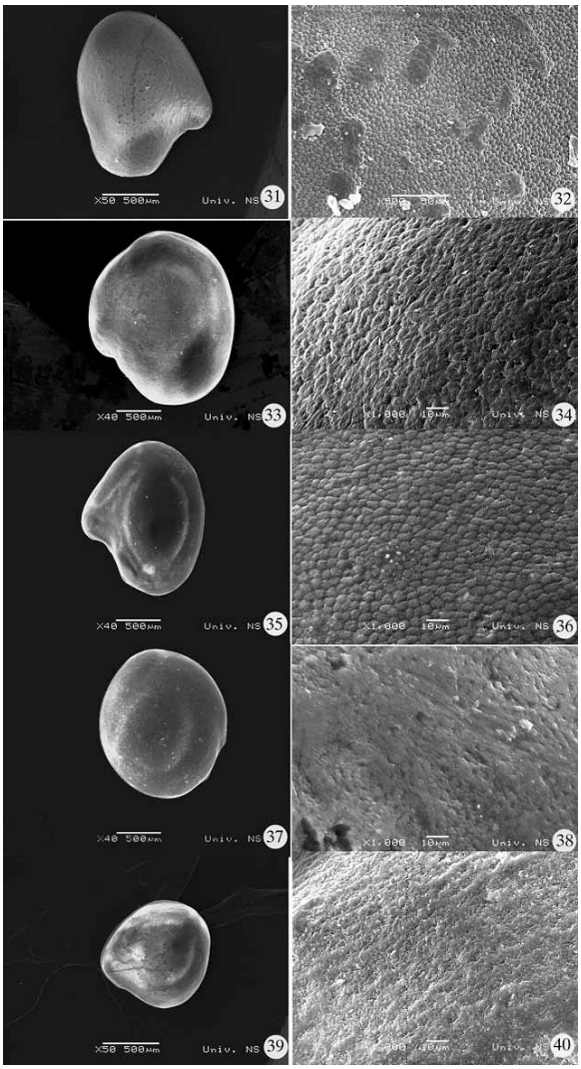

Figure 4. *Scanning electron micrographs of* Trifolium *seeds and details of seed coat surface. 31-32.* T. pallescens. *33-34.* T. pannonicum. *35-36.* T. pratense. *37-38.* T. purpureum. *39-40.* T. repens.

Seed coat surface

The surface of the seed coat may be smooth, roughened, papillose, tuberculate, reticulate or pitted (Figures 1–6). According to the seed coat characteristics, the species are separated into eight groups (Table 4). Only one species, *T. alexandrinum*, has pitted (umbillicatae) seed coat surface (Figures 1). The species of the second group (code 2) have smooth (glabrosae) surface, with flat periclinal walls of epidermal cells. The third group (code 3) comprises the species with roughened exotesta, with slightly convex periclinal walls of epidermal cells. The species of the fourth group (code 4) have papillose seed coat, where each papillae is made of one epidermal cell with strongly convex periclinal walls. Tuberculate seed coat is characteristic for the species of the fifth group (code 5). Each tubercle is made of several elongated and raised epidermal cells. The tubercles could be prominent and densely distributed, like in *T. retusum, T. strictum, T. trichopterum* and *T. vesiculosum* (Figures 5, 6), or smooth, in the form of shallow protuberances, like in *T. aureum, T. diffusum, T. pallescens* and *T. spadiceum* (Figures 1, 2, 4, 5). Both types of tubercles are arranged in an irregular fashion.

The species of the sixth group (code 6) have primary and secondary seed coat ornamentation. Besides primary tuberculate structure, with tubercles of different sizes and distribution, they also have secondary, papillose structure of exotesta. The seventh group (code 7) is composed of three species (*T. hybridum, T. medium* and *T. montanum*), which have papilloso-reticulate seed coat ornamentation (Figure 3). On the surface of their seeds, a dense network of variously oriented wrinkles is re-

TABLE 3

Quantitative seed characters of species from sections *Trifolium* and *Trichocephalum*.

Section	Species	seed length (μm)	seed width (μm)	seed index	weight of 1000 seeds (g)
Trifolium	*T. alexandrinum*	2310.1±54.9 (7.5)	1624.0±26.9 (5.2)	1.4±0.04 (8.3)	3.2±0.05 (2.5)
	T. alpestre	1762.9±23.5 (4.2)	1331.6±20.5 (4.9)	1.3±0.03 (6.9)	1.8±0.04 (3.3)
	T. angustifolium	1628.2±34.2 (6.6)	1185.0±20.8 (5.5)	1.4±0.04 (9.6)	1.4±0.02 (2.0)
	T. arvense	989.4±36.7 (11.7)	767.2±18.9 (7.8)	1.3±0.03 (6.9)	0,34±0.0 (0.0)
	T. dalmaticum	1614.1±26.4 (5.2)	892.6±11.4 (4.0)	1.8±0.03 (4.6)	0.5±0.0 (0.0)
	T. diffusum	1417.6±26.0 (5.8)	1219.4±16.9 (4.4)	1.2±0.02 (5.8)	1.3±0.01 (1.1)
	T. incarnatum	2489.8±111.1 (14.1)	1572.3±50.3 (10.1)	1.6±0.04 (8.8)	4.1±0.1 (4.3)
	T. medium	1931.1±28.6 (4.7)	1828.6±20.9 (3.6)	1.1±0.01 (4.3)	2.8±0.09 (5.3)
	T. pannonicum	2082.3±28.9 (4.4)	1773.7±26.6 (4.7)	1.2±0.0 (3.3)	3.6±0.5 (25.1)
	T. pratense	1819.2±27.4 (4.8)	1290.8±27.9 (6.8)	1.4±0.04 (8.6)	1.8±0.2 (14.6)
	T. purpureum	1727.2±45.2 (8.3)	1333.8±48.0 (11.4)	1.3±0.03 (0.9)	1.6±0.01 (1.1)
	T. rubens	1747.5±42.8 (7.7)	1438.4±38.7 (8.5)	1.2±0.03 (7.0)	2.4±0.4 (27.7)
	T. stellatum	1857.5±44.7 (7.6)	1425.7±39.6 (8.8)	1.3±0.03 (6.9)	2.4±0.05 (2.9)
	T. striatum	2000.3±21.4 (3.4)	1505.7±7.9 (1.7)	1.3±0.01 (3.2)	2.8±0.04 (2.0)
	T. trichopterum	1220.4±29.3 (7.6)	982.2±30.9 (9.9)	1.2±0.02 (5.4)	0.65±0.02 (4.3)
	average	1773.2±32.4 b (22.4)	1344.8±25.2 b (23.0)	1.3±0.02 ab (15.2)	2.2±0.2 b (38.5)
Trichocephalum	*T. subterraneum*	2237.3±88.0 a (12.4)	1880.8±76.5 a (12.9)	1.2±0.01 ab (2.4)	6.3±0.2 a (3.4)

TABLE 4

Distribution of characters and their states in seeds of *Trifolium* species.

Subgenus/Section		Species	length*	width	index	weight	color	shape	seed-coat surface	radicle lobe length	radicle lobe shape
Sect. *Lotoidea*	1	*T. alpinum*	4	4	2	4	4	2	3	2	2
	2	*T. angulatum*	1	1	2	1	4	2	8	3	2
	3	*T. hybridum*	1	1	1	1	1	4	7	2	2
	4	*T. montanum*	3	3	3	3	1	3	7	1	2
	5	*T. nigrescens*	1	1	2	1	1	2	3	2	2
	6	*T. ornithopodioides*	1	1	2	1	2	4	2	3	2
	7	*T. pallescens*	2	2	2	2	2	4	6	2	2
	8	*T. repens*	1	2	1	1	1	1	3	3	2
	9	*T. retusum*	1	1	1	1	1	4	6	2	2
	10	*T. thalii*	2	2	2	2	1	4	4	2	2
Sect. *Paramesus*	11	*T. strictum*	1	1	2	1	4	2	6	2	1
Sect. *Mistyllus*	12	*T. vesiculosum*	2	3	1	2	4	4	6	3	2
Sect. *Vesicastrum*	13	*T. fragiferum*	2	2	1	2	1	1	3	3	1
	14	*T. resupinatum*	2	2	2	2	2	2	2	3	1
Sect. *Chronosemium*	15	*T. aureum*	1	1	2	1	2	2	5	2	2
	16	*T. badium*	2	2	2	1	2	2	3	2	1
	17	*T. campestre*	1	1	3	1	1	3	2	2	1
	18	*T. dubium*	1	1	2	1	1	2	2	2	1
	19	*T. micranthum*	1	1	2	1	1	2	2	2	2
	20	*T. patens*	1	1	3	1	1	3	2	2	2
	21	*T. spadiceum*	1	1	3	1	2	3	5	1	2
	22	*T. velenovskyi*	1	1	2	1	2	2	2	2	2
Sect. *Trifolium*	23	*T. alexandrinum*	4	4	3	4	1	3	1	2	2
	24	*T. alpestre*	3	3	2	3	2	2	2	1	2
	25	*T. angustifolium*	3	2	2	2	1	2	2	2	2
	26	*T. arvense*	1	1	2	1	2	2	3	2	2
	27	*T. dalmaticum*	3	1	3	1	1	3	3	2	1
	28	*T. diffusum*	2	2	1	2	4	1	5	2	2
	29	*T. incarnatum*	4	3	3	4	1	3	3	2	1
	30	*T. medium*	3	4	1	4	1	4	7	1	2
	31	*T. pannonicum*	4	4	1	4	1	1	3	1	2
	32	*T. pratense*	3	2	3	3	1	3	4	1	2
	33	*T. purpureum*	3	3	2	3	1	2	3	2	1
	34	*T. rubens*	3	3	2	4	1	2	3	2	2
	35	*T. stellatum*	3	3	2	4	1	2	2	2	1
	36	*T. striatum*	4	3	2	4	1	2	2	2	1
	37	*T. trichopterum*	1	1	2	1	1	2	6	3	1
Sect. *Trichocephalum*	38	*T. subterraneum*	4	4	1	4	3	1	2	2	2

* Length: (1) 0.90–1.25mm, (2) 1.26–1.60mm, (3) 1.61–1.95mm, (4) >1.95mm. Width: (1) 0.70–1.00mm, (2) 1.01–1.30mm, (3) 1.31–1.60mm, (4) >1.60 mm. Index: (1) 1.00–1.19, (2) 1.20–1.39, (3) >1.40. Weight of 1000 seeds: (1) 0.20–0.80g, (2) 0.81–1.40g, (3) 1.41–2.00g, (4) > 2.00g. Color: (1) yellow to brown, (2) yellow to brown, green, (3) purple, (4) brown. Shape: (1) round, (2) ovoid, (3) elongated ovoid, (4) heart-shaped. Seed coat surface: (1) pitted, (2) smooth, glabrous (3) roughened, (4) papillose, (5) tuberculate, (6) papilloso-tuberculate, (7) papilloso-reticulate, (8) papilloso-tuberculato-reticulate. Radicle lobe length: (1) radicle lobe up to 1/2 of cotyledon lobe length, (2) radicle lobe up to 2/3 of cotyledon lobe length, (3) radicle lobe of almost the same length as the cotyledon lobe. Radicle lobe shape: (1) tightened, (2) prominent.

corded. *T. angulatum* is the only species in the eight group, with its papilloso-tuberculato-reticulate seed coat surface (Figure 1).

The epidermal cells are polygonal in shape for all examined species, with flat or slightly sinuous anticlinal walls.

Principal Components Analysis (PCA) and Multiple Correspondence Analysis (MCA)

Principal Components Analysis (PCA) indicates three groups of characters, which explain 71.05% of the total variation (Table 5). The first principal component explains 34.35% of the variation. It is defined by quantitative characters, such as seed length, width and weight, which contribute most to the total variation. The second principal component explains 18.97% of variation. The third component, which explains 17.73% of variation, is defined by the seed shape.

The results of Multiple Correspondence Analysis show that the species of the section *Chronosemium* are most similar to each other, on the basis of their seed characters, and they form a relatively homogenous group (Figure 7). Other sections, especially section *Trifolium,* show more heterogeneity, and do not form separate groups. The species with the most distinguishing seed characters is *T. subterraneum* (Figure 5, 7). The seeds of this species have purple color, very large size and the highest weight. The combination of other examined characteristics is also unique: seeds round in shape, with prominent radicle lobe of up to 2/3 of the cotyledon lobe length, with smooth seed coat surface and flat periclinal walls of epidermal cells. These outstanding features of *T. subterraneum* seeds could be explained by the specific conditions of ripening of fruiting heads and seed development under ground.

Another species with a unique combination of characters is *T. vesiculosum*. The seeds of this species are of medium size, brown, heart-shaped, with prominent radicle lobe of almost the same length as the cotyledon lobe. They have seed coat with very prominent, densely distributed tubercles and papillose periclinal walls of epidermal cells.

Two species could be easily identified on the basis of their unique ornamentation of seed coat surface. *T. angulatum* could be singled out by its papilloso-tuberculato-reticulate ornamentation, and *T. alexandrinum* is the only species with pitted seed coat (Figure 1, 7).

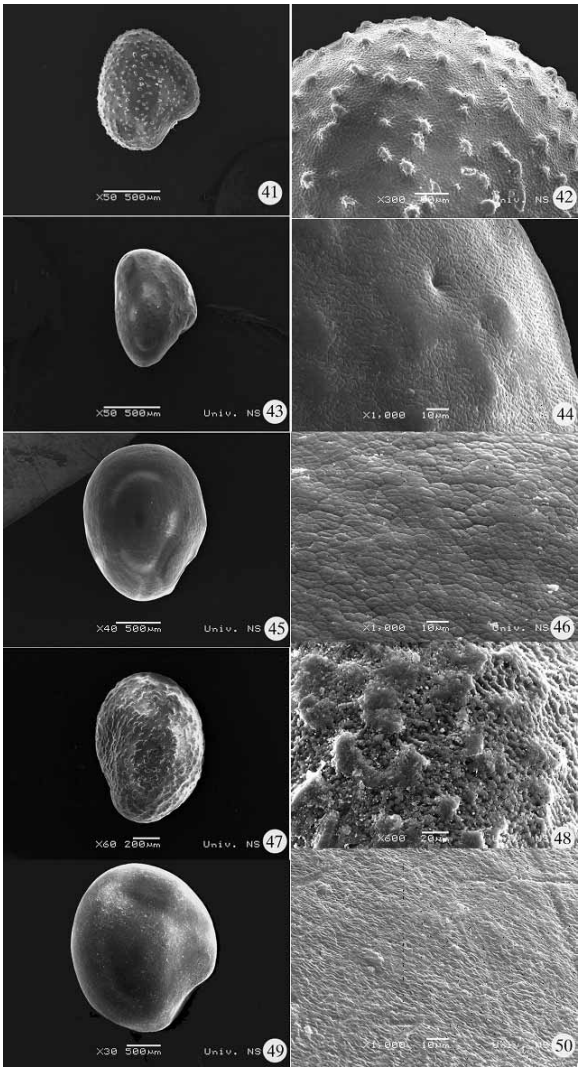

Figure 5. *Scanning electron micrographs of* Trifolium *seeds and details of seed coat surface. 41-42.* T. retusum. *43-44.* T. spadiceum. *45-46.* T. stellatum. *47-48.* T. strictum. *49-50.* T. subterraneum.

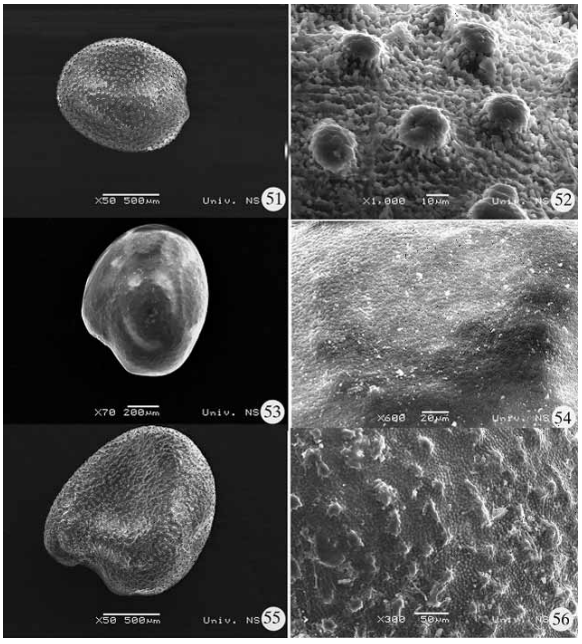

Figure 6. *Scanning electron micrographs of* Trifolium *seeds and details of seed coat surface. 51-52.* T. trichopterum. *53-54.* T. velenovskyi. *55-56.* T. vesiculosum.

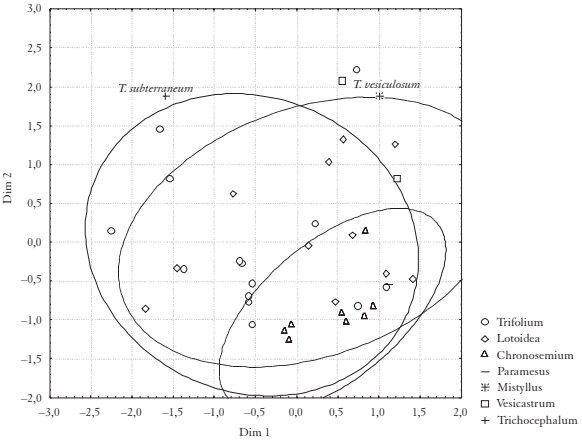

Figure 7. *Plot of Multiple Correspondence Analysis (MCA) results. (Ellipses represent the range of 90% of variation for sections* Lotoidea, Trifolium *and* Chronosemium*).*

However, a large number of the examined species could not be separated on the basis of their seed characteristics. Two groups of species with similar or even identical combination of seed characteristics are defined. *T. nigrescens, T. micranthum, T. dubium, T. velenovskyi* and *T. arvense* have very small seeds (1.0–1.1 × 0.7–0.9 mm) of ovoid shape, with seed index values of 1.25–1.35, with prominent radicle lobe of up to 2/3 of the cotyledon lobe length, yellow to brown or green color, with smooth, or slightly papillose seed coat surface (Figure 1, 2, 3, 6). The other group comprised species with seeds of medium size (1.6–1.9 × 1.2–1.4 mm), index value of 1.22–1.38, of ovoid shape, with more or less prominent radicle lobe of up to 2/3 of the cotyledon lobe length, and smooth or slightly papillose seed coat surface (*T. alpestre, T. angustifolium, T. purpureum, T. rubens* and *T. stellatum*) (Figure 4, 5). The scatterplot of MCA results (Figure 7) also shows that ellipses, which represent the range of variation of 95% of samples of each section, overlap, which means that subgenera or sections could not be separated on the basis of the seed morphological and micromorphological characters. Additional characters are needed for further determination of *Trifolium* species.

TABLE 5

Principal Components Analysis (PCA) of seed morphological traits. Factor coordinates of the variables, based on correlations (marked loadings are >0.700) and cumulative percentages of the vectors.

	Factor 1	Factor 2	Factor 3
length	0.957789*	0.000852	–0.023907
width	0.928768*	0.262514	–0.084169
index	0.035123	–0.655097	0.454847
weight	0.962116*	0.112095	–0.004164
color	–0.145047	0.642539	–0.326532
shape	–0.183299	0.090301	0.766149*
radicle lobe length	–0.472357	0.018732	–0.628176
radicle lobe shape	0.001894	0.596888	0.410520
seed coat surface	–0.327533	0.647270	0.352109
cumulative percentages of the vectors	34.3546	53.3238	71.0491

DISCUSSION

Variation in *Trifolium* seed morphology is manifested mainly in seed size, shape, color, seed coat ornamentation and morphology of the radicle lobe. Among the examined parameters, seed length, width and weight are strongly positively correlated, as well as seed index and seed shape. Quantitative characteristics, connected to the seed size, contribute most to the total variation. Zohary and Heller *(11)* described five, while Taia *(13)* only three types of seed coat patterns. According to our observations, the seed coat patterns were more variable, and by more detailed analysis, we increased the number of character states and distinguished eight different patterns. Taia *(13)* reports only oval and global shape of the *Trifolium* seeds, while, according to our observations, besides those basic shapes, the seeds could also be characterized as elongated-ovoid and heart-shaped.

The results of our analyses showed that the examined characters could not provide considerable information that could be used to distinguish sections of this genus. Seed features do not support the infrageneric classification. The seeds of the species of the sect. *Chronosemium* show the smallest differences among the species. Most of them are of small size, ovoid or elongated ovoid in shape, with more or less prominent radicle lobe of up to 2/3 of the cotyledon lobe length. Sect. *Lotoidea* and *Trifolium* show a wide range of variability among the species. Generally, but not obligatory, species of sect. *Lotoidea* have seeds of smaller size, with longer radicle lobe, while species of sect. *Trifolium* have larger seeds, with shorter radicle lobe.

A unique seed coat morphology and combination of other seed morphological and micromorphological characteristics permits easy identification of *T. subterraneum, T. alexandrinum, T. angulatum* and *T. vesiculosum*. Despite this, a large number of examined species could not be identified on the basis of the seed morphology. Two major groups of species are defined with extremely similar external appearance, so that is impossible to distinguish between them. The species of the first group are *T. nigrescens, T. micranthum, T. dubium, T. velenovskyi* and *T. arvense,* while *T. alpestre, T. angustifolium, T. purpureum, T. rubens* and *T. stellatum* join the second group. Slight differences in seed morphological and micromorphological features indicate the need for further studies on other morphological traits in order to facilitate determination within these groups.

Acknowledgements: *This work was financially supported by the Ministry of Science and Technological Development, Republic of Serbia, Grant No. 143037. The authors would like to thank Mr. Milos Bokorov from the University Center for Electron Microscopy, Novi Sad, Serbia, for his technical assistance and SEM microscopy. We would also like to thank the University of Kentucky, Department of Plant and Soil Science, College of Agriculture, Lexington, Kentucky; Universite Joseph Fourier, UFP de Biologie, Grenoble, France and Institute of Plant Genetics and Crop Plant Research, Genebank, Gatersleben, Germany for providing seed samples from their collections.*

REFERENCES

1. ALGAN G, BÜYÜKKARTAL H N B 2000 Ultrastructure of the seed coat development in the natural tetraploid *Trifolium pratense* L. *J Agron Crop Sci 184*: 205–213
2. SEGGARA J G, MATEU I 2001 Seed morphology of *Linaria* species from eastern Spain: identification of species and taxonomic implications. *Bot J Linn Soc 135*: 375–389
3. BOBROV A V F C, MELIKIAN A P, ROMANOV M S, SOROKIN A N 2004 Seed morphology and anatomy of *Austrotaxus spicata* (*Taxaceae*) and its systematic position. *Bot J Linn Soc 145*: 437–443
4. HASSAN N M S, MEVE U, LIEDE-SCHUMANN S 2005 Seed coat morphology of *Aizoaceae-Sesuvioideae, Gisekiaceae* and *Molluginaceae* and its systematic significance. *Bot J Linn Soc 148*: 189–206
5. MARIN P, BOZA P, MERKULOV LJ, KRSTIC B, PETKOVIC B, VELJIC M, PAJEVIC S 1998 Seed sculpturing of selected European *Vicia* L. species (*Fabaceae*) and their taxonomic evaluation. *Seed Sci Tech 26*: 17–32
6. JUAN R, PASTOR J, FERNANDEZ I 2000 SEM and light microscope observations on fruit and seeds in *Scrophulariaceae* from Southwest Spain and their systematic significance. *Ann Bot 86*: 323–338
7. MORO F, PINTO A C R, DOS SANTOS J M, DAMIAO FILHO C F 2001 A scanning electron microscopy study of the seed and post-seminal development in *Angelonia salicariifolia* Bonpl. (*Scrophulariaceae*). *Ann Bot 88*: 499–506
8. ANISZEWSKI T, KUPARI M H, LEINONEN A J 2001 Seed number, seed size and seed diversity in Washington Lupin (*Lupinus polyphyllus* Lindl.). *Ann Bot 87*: 77–82
9. BONILLA-BARBOSA J, NOVELO A, HORNELAS OROZCO J, MARQUEZ-GUZMAN J 2000 Comparative seed morphology of Mexican *Nymphaea* species. *Aquat Bot 68*: 189–204
10. ESAU C 1977 Anatomy of Seed Plants. John Wiley and Sons, New York/Santa Barbara/London/Sydney/Toronto.
11. ZOHARY M, HELLER D 1984 The Genus Trifolium. The Israel Academy of Sciences and Humanities, Jerusalem.
12. SLATTERY H D, ATWELL B J, KUO J 1982 Relationship between color, phenolic content and impermeability in the seed coat of various *Trifolium subterraneum* L. genotypes. *Ann Bot 50*: 376–378
13. TAIA W 2004 Tribe *Trifolieae*: Evidence from seed characters. *Pak J Biol Sci 7 (7)*: 1287–1302
14. GUNN C R 1981 Seed topography in the *Fabaceae*. *Seed Sci Tech 9*: 737–757
15. STEARN W T 1992 Botanical Latin, 4th Edition. Timber Press, Oregon, USA.
16. STATSOFT INC. 2004. STATISTICA (data analysis software system), version 7. www.statsoft.com.