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Micromorphological study of some *Trifolium* taxa (*Fabaceae*) seeds from Greece and its systematic significance

Abstract

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Greece is considered a diversity center for the genus *Trifolium* L. (*Fabaceae*), which is represented in the country by 95 taxa. However, the classification of the genus may vary according to different taxonomic approaches. The current study aims to contribute to the study of the genus by describing the seed coat surface of 23 *Trifolium* taxa that were collected in various localities of southern Greece. The seeds were obtained from herbarium specimens and observed with Scanning Electron Microscope.

Key words: SEM, taxonomy, *Faboideae*, biosystematics.

Introduction

The genus *Trifolium* L. (*Fabaceae*) consists of approximately 255 species (Smýkal & al. 2015; Scoppola & al. 2018), distributed throughout the temperate and subtropical regions of the world, even though representatives of the genus can also be found restricted to the montane and alpine zones in the tropics of West Africa and South America (Zohary & Heller 1984). Greece, along with Anatolia, constitute a diversity center for the genus *Trifolium* (Zohary & Heller 1984), which is represented in the country by 95 taxa (species and subspecies), including 8 endemic ones (Dimopoulos & al. 2013). The number of *Trifolium* taxa frequently varies among the authors depending on their taxonomical approach, the progress of botanical explorations and the increase of systematic and evolutionary studies (Samaropoulou & al. 2019).

Trifolium taxa in Greece occur in various types of natural, semi-natural and anthropogenic habitats, generally at places that are exposed to high solar radiation (Ellison & al. 2006; Samaropoulou & al. 2019).

A significant number of publications are dealing with the seed coat morphological and micromorphological characteristics of various families and their systematic implications. Among the first relative studies concerning genera of the *Fabaceae* include those of Lersten (1979, 1981), Lersten & Gunn (1982), Manning & van Staden (1987) and Small & al. (1990).

In the framework of the revision of the genus *Trifolium*, Zohary & Heller (1984) examined the seed coat surface of 24 species, providing relative SEM photographs for 8 of them and they described 5 different types of seed coat patterns. According to Pinar & al. (2001) diploid and tetraploid forms of *Trifolium pratense* L. can be distinguished by their seed coat ornamentation. Taia (2004) examined eight *Trifolium* species and recognised three seed coat patterns, while Salimpour & al. (2007) studied the seed morphology of seven *Trifolium* taxa from Iran. Zorić & al. (2010) studied 38 *Trifolium* species and separated them into eight groups based on seed coat morphological characters.

However, the study of seed characteristics of this taxonomically, ecologically and economically important genus remains incomplete especially concerning Greek taxa. The present study aims to contribute to the taxonomy of the genus *Trifolium* by describing the seed coat surface patterns of 23 taxa from Greek populations.

Materials and Methods

Material was obtained from herbarium specimens collected by the first author from various localities of southern Greece and deposited in ACA (Table 1). The seeds were put directly onto carbon adhesive discs on aluminum stubs and sputter coated with approximately 25 nm of gold. The observation was carried out with a FEI Quanta Inspect SEM and it took place in the Electron Microscopy and Nanomaterials Laboratory, Department of Materials Science, Demokritos Institute. At least 15 seeds of each taxon were analysed and seed length and width, aspect ratio (L/W), shape and coat surface are given.

The determination of the seed shape follows Zorić & al. (2010) and takes into consideration aspect ratio and the length of the radical lobe. Seeds are characterised as round (aspect ratio up to 1.19), ovoid (aspect ratio 1.20-1.39), elongated-ovoid (aspect ratio more than 1.40) and, in the case of the radicle lobe being very prominent, heart-shaped.

The terminology of the coat surface follows Lersten (1981), Lersten & Gunn (1982), Manning & van Staden (1987) and Small & al. (1990).

Results and Discussion

The present study examines the seed micromorphological features in 23 taxa of the genus *Trifolium* including representatives from the major sections with Mediterranean floristic elements. Seed morphometric characters, shape and coat pattern is reported for the first time for 10 species, among them, the Greek endemic *Trifolium aurantiacum*. At least x5000 magnification is required in order to obtain a precise idea of the seed surface micromorphology and avoid inconsistencies among various reports concerning the same species. As mentioned by Lersten (1979), seeds of most *Fabaceae* are smooth and featureless at low magnification. The seed coat surface micromorphology of the examined taxa is demonstrated in Figs 1-6.

The seeds of the studied taxa vary in size from 0.8803 mm to 2.3333 mm in length and from 0.6092 mm to 1.9245 mm in width. Although *Trifolium subterraneum* has the largest seed dimensions measured, its aspect ratio is one of the smallest ones (1.1458). However,

Table 1. List of examined taxa.

Taxon	Locality	Voucher No
<i>Trifolium angustifolium</i> L.	Attiki, Mt. Pendelikon, hills N of Dionisovouni summit, near the chapel of Agios Ioannis, road margins, 490 m, 4 Jun 2018	12929
<i>Trifolium arvense</i> L.	Attiki, municipality of Thrakomakedones, ca. 1 km ESE of the monastery of Kimiseos Theotokou, recently burnt <i>Pinus halepensis</i> Mill. forest, coarse-grained fluviolacustrine formations on Parnitha piedmont, 350 m, 4 May 2018	12908
<i>Trifolium aurantiacum</i> Boiss. & Spruner	Peloponnisos, Mt. Aphrodisio, ca. 0.5 km SE of Dechouni village, <i>Quercus coccifera</i> L. scrub, limestones, 850 m, 24 May 2013	8154
<i>Trifolium boissieri</i> Guss.	Kiklades, the island of Kea, path margins on the way to Karthaia archaeological site, 50 m, 27 May 2018	12680
<i>Trifolium campestre</i> Schreb.	Attiki, municipality of Thrakomakedones, ca. 1 km ESE of the monastery of Kimiseos Theotokou, recently burnt <i>Pinus halepensis</i> Mill. forest, coarse-grained fluviolacustrine formations on Parnitha piedmont, 350 m, 4 May 2018	12906
<i>Trifolium cherleri</i> L.	Peloponnisos, Mt. Aphrodisio, ca. 1 km S of Nasia village, forest roadsides, 700-800 m, 26 May 2013	8699
<i>Trifolium fragiferum</i> L.	Attiki, Stamata village, near the chapel of Agia Paraskevi, damp places by <i>Platanus orientalis</i> L. stream, 350 m, 12 Jul 2007	4000
<i>Trifolium glomeratum</i> L.	Peloponnisos, Mt. Aphrodisio, ca. 1 Km S of Nasia village, <i>Quercus frainetto</i> Ten. forest and roadsides, radiolarites, 700-800 m, 26 May 2013	8749
<i>Trifolium grandiflorum</i> Schreb.	Kiklades, the island of Kea, path margins on the way to Karthaia archaeological site, 20-100 m, 27 May 2018	12678
<i>Trifolium hirtum</i> All.	Attiki, Mt. Pendelikon, in the locality Chrisouli Rachi, schistose slopes with phrygana, 650 m, 19 May 2002	858
<i>Trifolium lappaceum</i> L.	Peloponnisos, Mt. Aphrodisio, between Kondovazena and the crossroad to Peleki village, damp places by the roadsides, 650 m, 25 May 2013	8567
<i>Trifolium leucanthum</i> M. Bieb.	Peloponnisos, Mt. Aphrodisio, ca. 1 km S of Nasia village, forest roadsides, 700-800 m, 26 May 2013	8698
<i>Trifolium nigrescens</i> Viv.	Kiklades, the island of Kea, path margins on the way to Karthaia archaeological site, 20-100 m, 27 May 2018	12682
<i>Trifolium patens</i> Schreb.	Peloponnisos, Mt. Aphrodisio, between Dafni and the crossroad to Nasia, roadsides, 550-600 m, 26 May 2013	8645
<i>Trifolium physodes</i> M. Bieb.	Attiki, Mt. Pendelikon, near the water tanks of Dionisos municipality, path margins in densely regenerated <i>Pinus halepensis</i> Mill. forest, 550 m, 3 Jun 2007	3773
<i>Trifolium pignantii</i> Fauché & Chaub.	Peloponnisos, Mt. Aphrodisio, between Pera Vachlia village and the dam of Ladona lake, <i>Quercus frainetto</i> Ten. forest, mainly radiolarites, 500 m, 25 May 2013	8633
<i>Trifolium repens</i> L.	Peloponnisos, Mt. Aphrodisio, Dechouneika village, 620 m, walnut orchard, 620 m, 24 May 2013	8066
<i>Trifolium resupinatum</i> L. subsp. <i>resupinatum</i>	Peloponnisos, Mt. Aphrodisio, between Dafni and the crossroad to Nasia village, roadsides, 550-600 m, 26 May 2013	8673
<i>Trifolium stellatum</i> L.	Peloponnisos, Mt. Aphrodisio, ca. 0.5 km SE of Dechouni village, forest roadsides, 850 m, 24 May 2013	8164

Table 1. continued.

<i>Trifolium striatum</i> L.	Peloponnisos, Mt. Aphrodisio, between Dechouneika village and the sanctuary of Aphrodite, road cuttings, 800 m, 26 May 2013	8822
<i>Trifolium subterraneum</i> L.	Peloponnisos, Mt. Aphrodisio, ca. 1 Km S of Nasia village, forest roadsides, 700-800 m, 26 May 2013	8755
<i>Trifolium suffocatum</i> L.	Attiki, Mt. Pendelikon, northern slopes of Megali Mavrinora, sparse <i>Quercus coccifera</i> L. scrub, marbles, 550 m, 14 May 2008	4195
<i>Trifolium tomentosum</i> L.	Attiki, NW part of Mt. Pendelikon, path margins near the chapel of Profitis Ilias, 550 m, 10 May 2003	1378

the smallest value of aspect ratio (1.1206) are observed in *T. suffocatum*. The longest seeds are observed in *T. stellatum* (2.3333 mm) which has one of the biggest aspect ratio (1.5908), forming elongated-ovoid seeds, while the most elongated seeds belong to *T. cherleri* (aspect ratio 1.6243). Both *T. repens* and *T. subterraneum* are characterised by a small aspect ratio with a prominent radical lobe, which makes them heart shaped (Fig. 5A1 and Fig. 6A1 respectively).

The main types of seed surface patterns recognised are the following: foveolate, reticulate, papillose, rugulate (rugose). Patterns with mixed characters also exist (Table 2).

One of the most common patterns observed is the “foveolate”. However, this pattern is very diversified and, in some cases, obscured, observed only at a higher magnification. Each of the species belonging to this group of “foveolate” has a distinctive micromorphology. *T. arvense* and *T. nigrescens* exhibit the highest similarity in their seed coat surface characteristics. Together with *T. grandiflorum* they constitute a quite homogeneous subgroup within this type of pattern. The similarities of the aforementioned taxa though, are not justified by their affinities, as proposed by various infrageneric classifications. They are distantly related, as they belong to different sections as defined by Zohary & Heller (1984).

T. hirtum, *T. pignantii* and *T. angustifolium* constitute another subgroup within this type of pattern. These species belong to the same section, but in different subsections (Zohary & Heller 1984). In addition, seed coat surface of *T. hirtum* and *T. pignantii* exhibits many similarities with the one presented in Zohary & Heller (1984) for *Trifolium microcephalum* Pursh, even though the latter species is native to western North America and belongs to section *Involucrarium* Hook., which includes only American species (Zohary & Heller 1984).

T. hirtum and *T. cherleri* are closely related taxa, but they have very distinct seed coat patterns and they are easily distinguished based on this criterion. The same also applies for *Trifolium repens* and *T. nigrescens*.

On the other hand, the similarities observed in the seed coat surface of *T. tomentosum* and *T. physodes* justify their affinities proposed by classical methods.

T. aurantiacum, which is considered a Greek endemic (Dimopoulos & al. 2013), has a peculiar pattern. The papillae of the seed surface are obscurely discerned owing to the overlying cuticular reticulum.

Some unusual micromorphological features that are observed in the examined species enhance the deviation from the patterns which have been described by now and increase considerably their diversity. These features are a criterion to distinct the investigated taxa

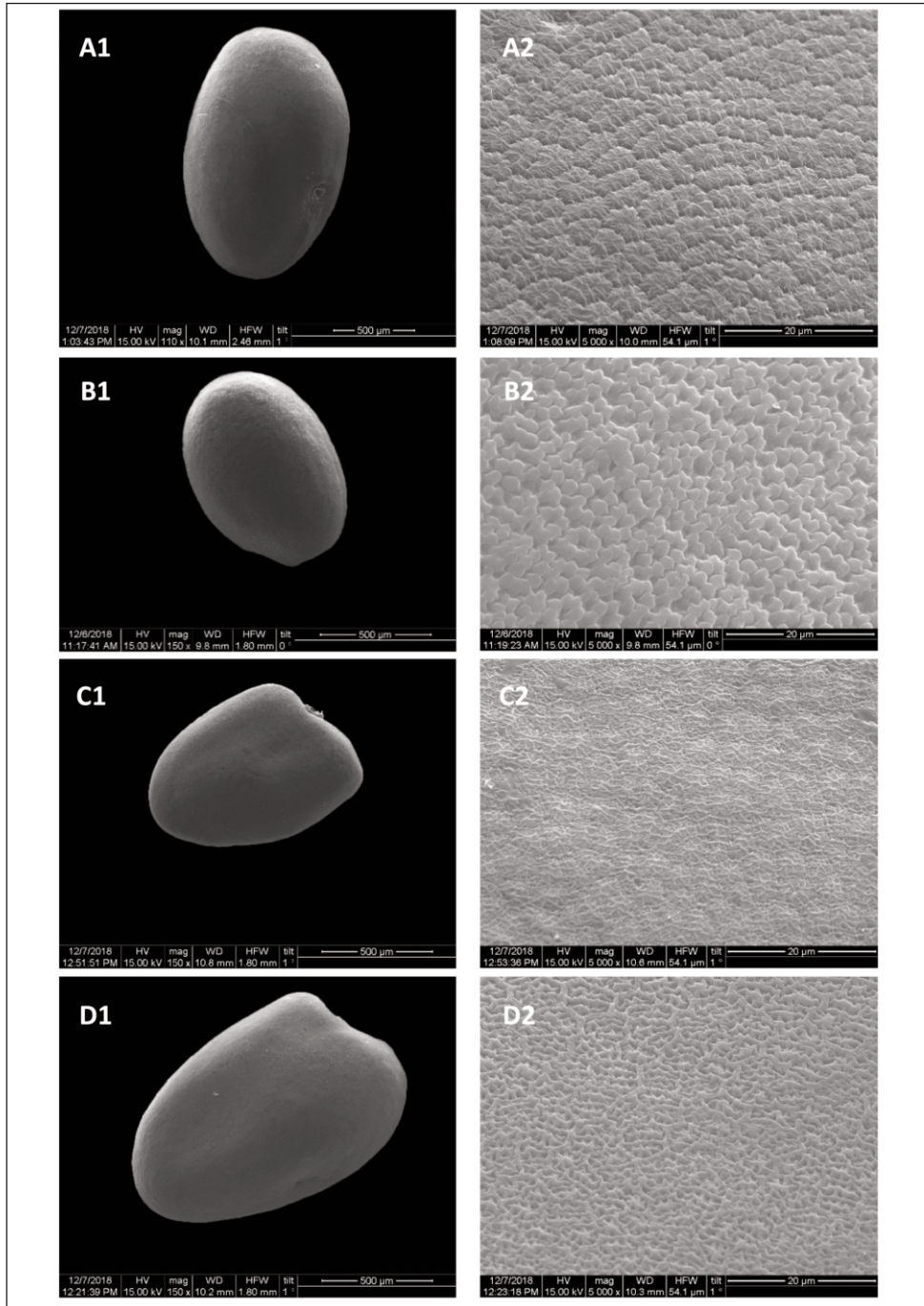


Fig. 1. 1. Seed overview; 2. seed coat surface micromorphology of: A) *Trifolium angustifolium*; B) *T. arvense*; C) *T. aurantiacum*; D) *T. boissieri*.

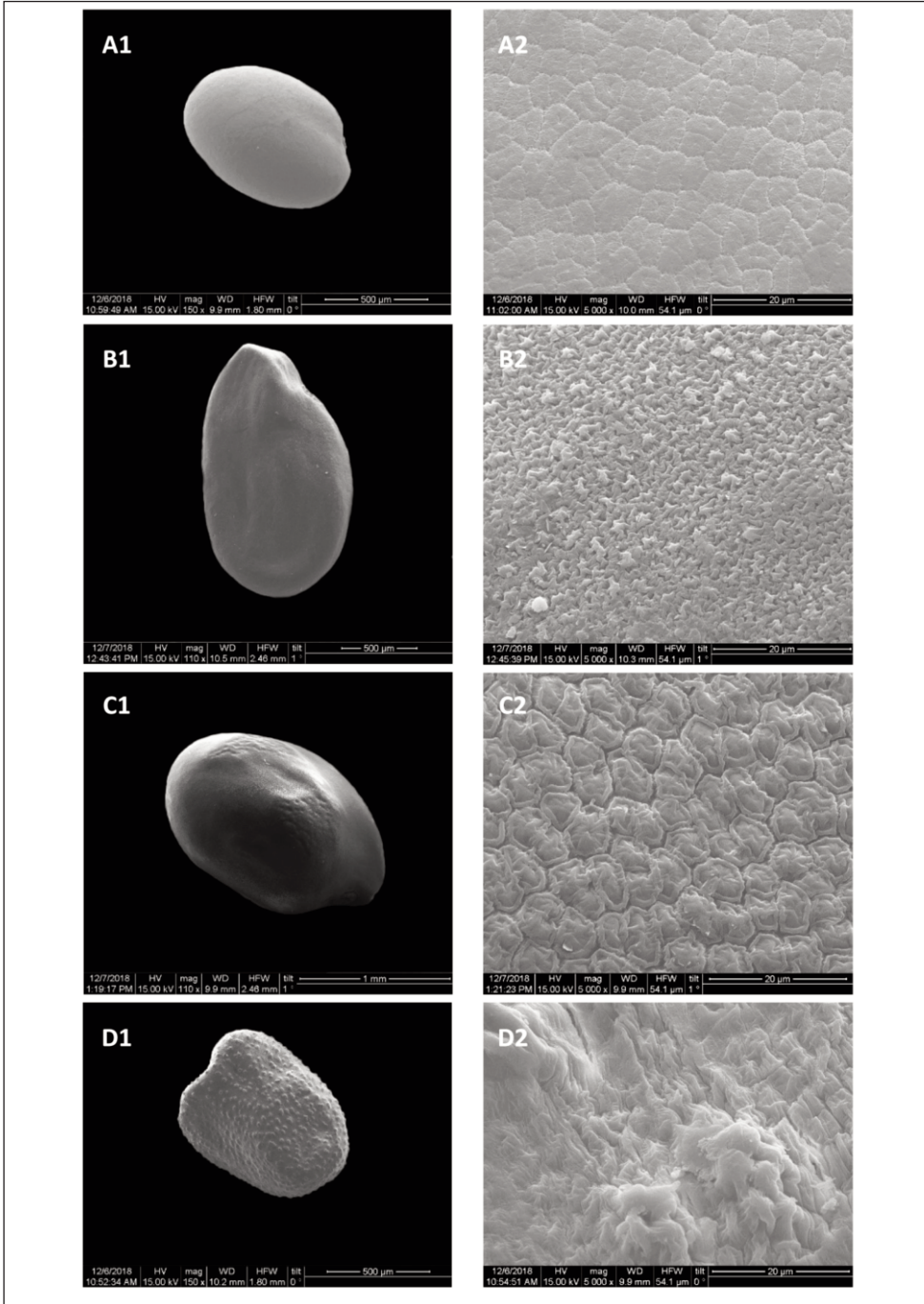


Fig. 2. 1. Seed overview; 2. seed coat surface micromorphology of: A) *Trifolium campestre*; B) *T. cherleri*; C) *T. fragiferum*; D) *T. glomeratum*.

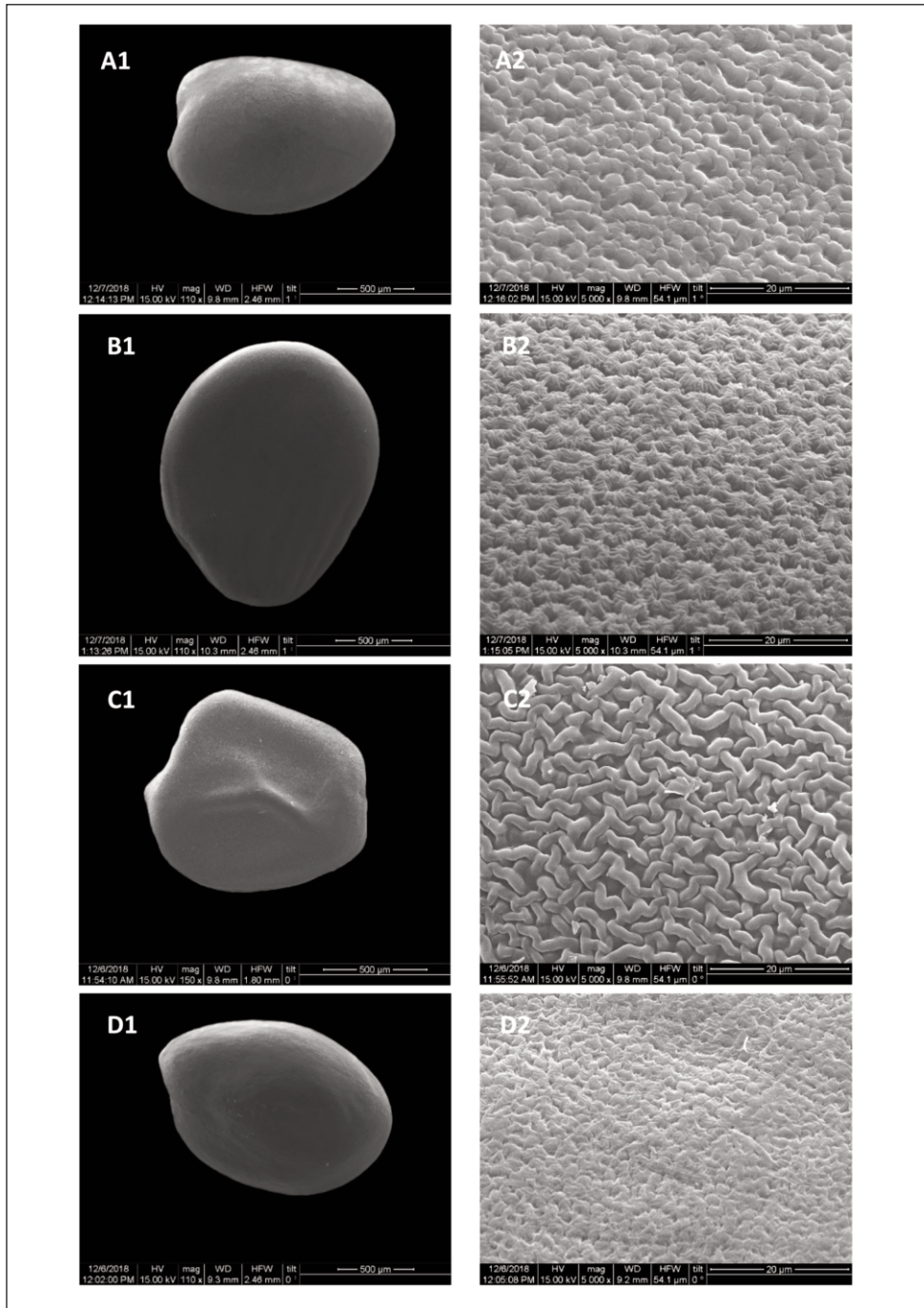


Fig. 3. 1. Seed overview; 2. seed coat surface micromorphology of: A) *Trifolium grandiflorum*; B) *T. hirtum*; C) *T. lappaceum*; D) *T. leucanthum*.

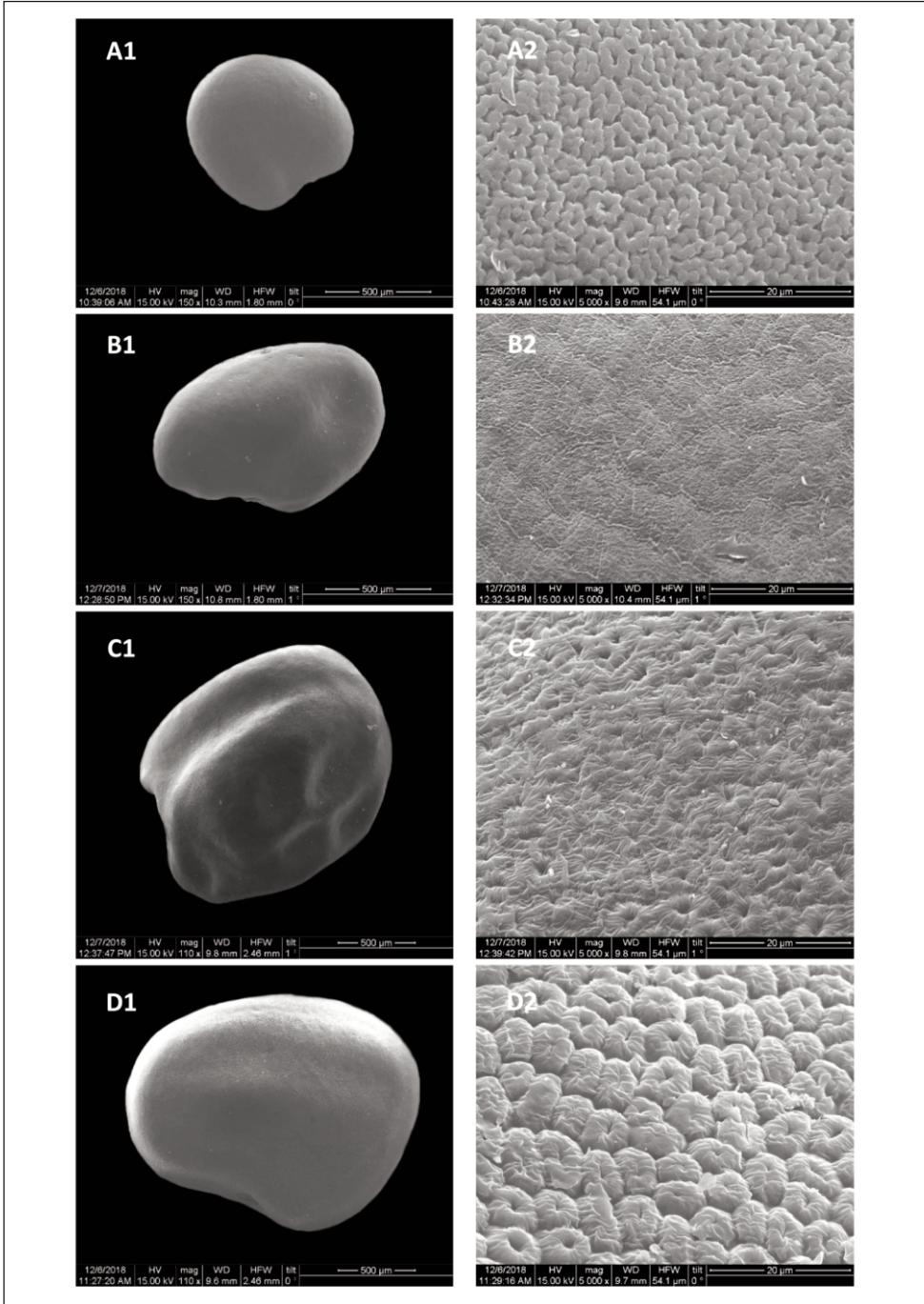


Fig. 4. 1. Seed overview; 2. seed coat surface micromorphology of: A) *Trifolium nigrescens*; B) *T. patens*; C) *T. physodes*; D) *T. pignanti*.

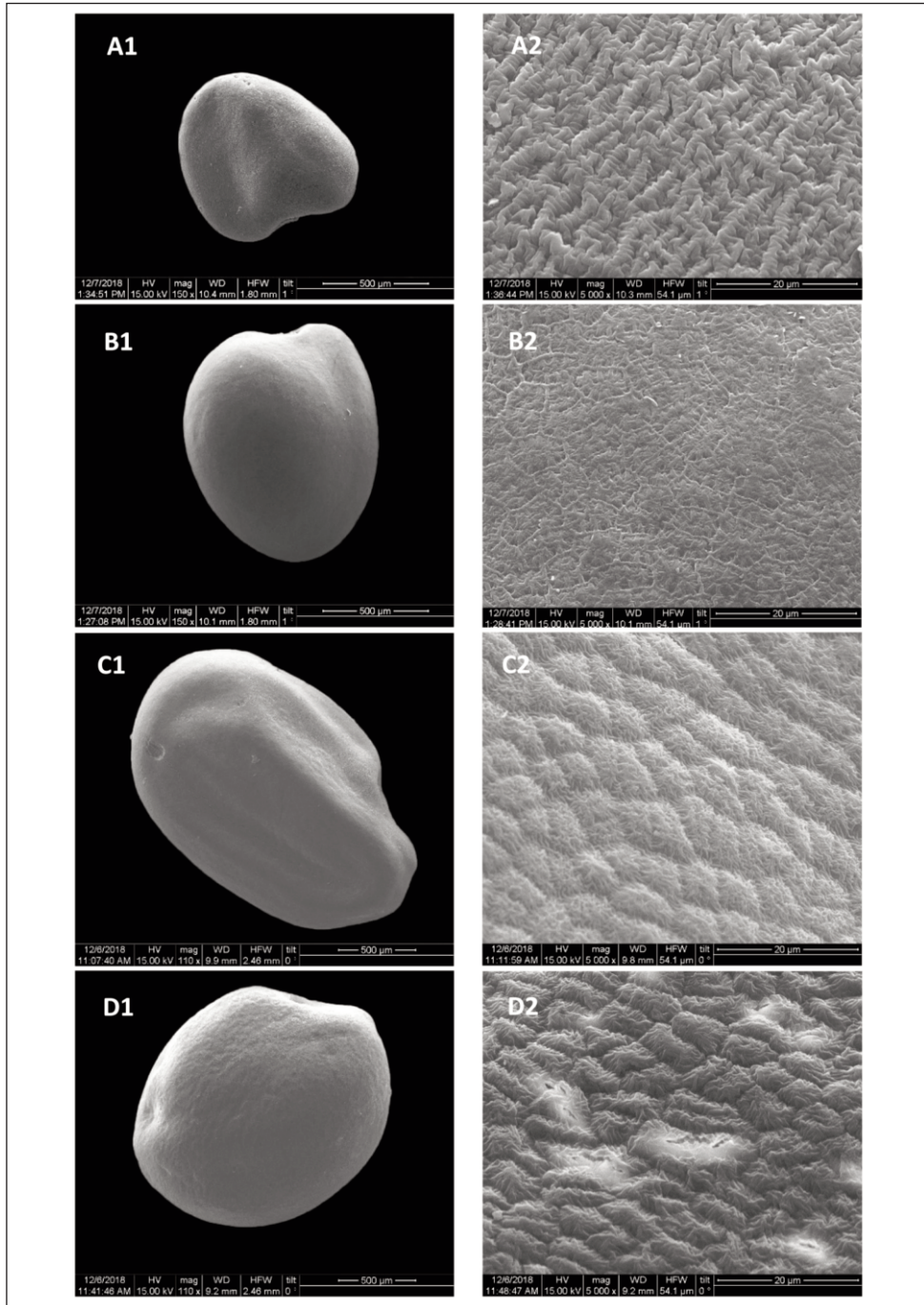


Fig. 5. 1. Seed overview; 2. seed coat surface micromorphology of: A) *Trifolium repens*; B) *T. resupinatum* subsp. *resupinatum*; C) *T. stellatum*; D) *T. striatum*.

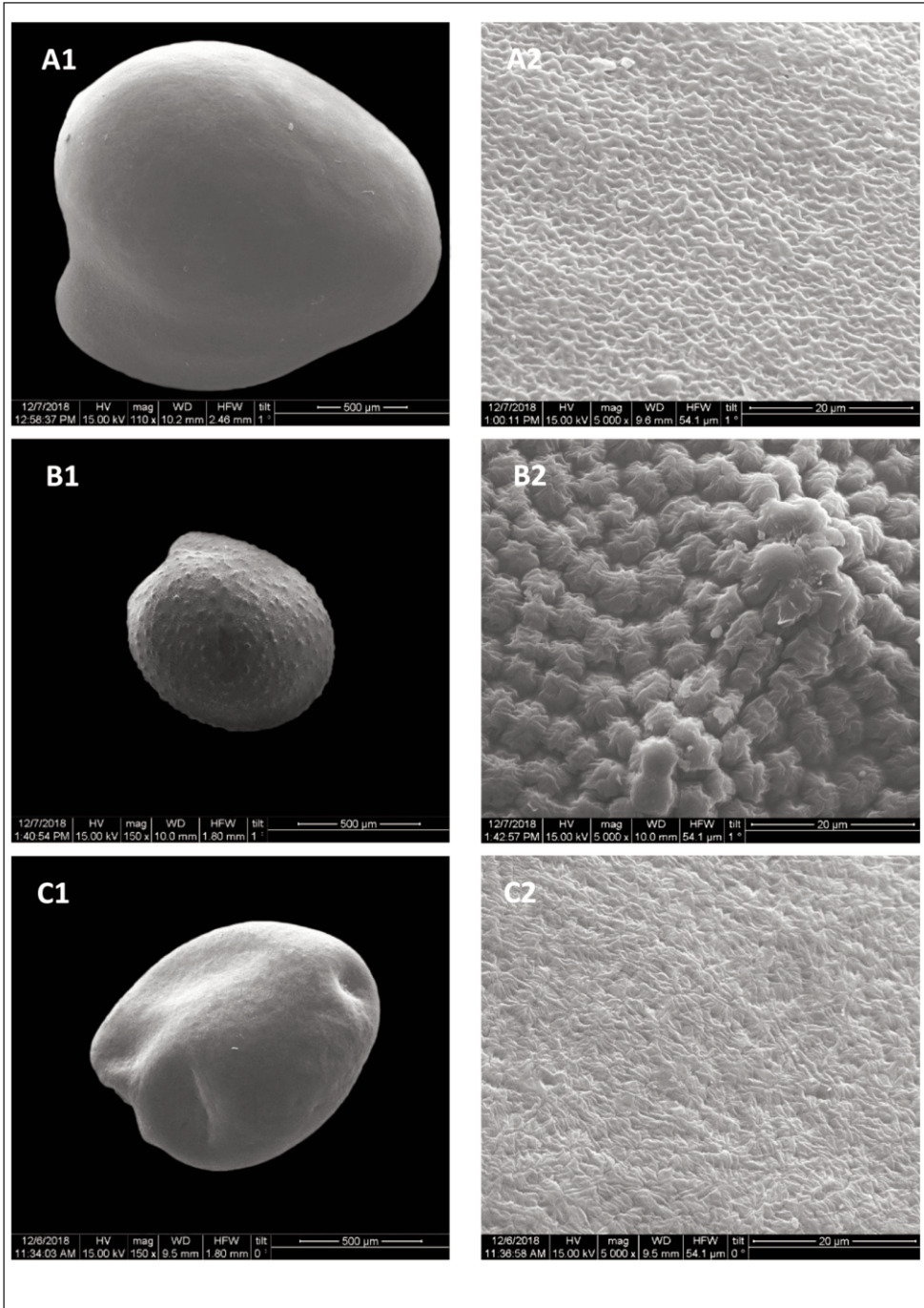


Fig. 6. 1. Seed overview; 2. seed coat surface micromorphology of: A) *Trifolium subterraneum*; B) *T. suffocatum*; C) *T. tomentosum*.

Table 2. Seed characters of *Trifolium* species. The mean value of length and width in mm is given along with standard deviation (SD). Taxa studied for the first time are indicated with an asterisk (*).

Taxon	Length (SD)	Width (SD)	Aspect ratio (L/W)	Seed shape	Seed coat surface
<i>T. angustifolium</i>	1.4881 (0.0894)	1.0127 (0.0304)	1.4695	Elongated-ovoid	Foveolate (obscurely)
<i>T. arvense</i>	0.9053 (0.0171)	0.6388 (0.0422)	1.4172	Elongated-ovoid	Foveolate
* <i>T. aurantiacum</i>	0.9596 (0.0792)	0.6793 (0.0649)	1.4126	Elongated-ovoid	Low papillose with overlying cuticular reticulum
* <i>T. boissieri</i>	0.9044 (0.1281)	0.7666 (0.1476)	1.1798	Round	Multi-reticulate
<i>T. campestre</i>	0.8904 (0.0263)	0.6092 (0.0085)	1.4616	Elongated-ovoid	Foveolate (obscurely)
* <i>T. cherleri</i>	1.6133 (0.0812)	0.9932 (0.0154)	1.6243	Elongated-ovoid	Rugulate
<i>T. fragiferum</i>	1.6157 (0.0209)	1.2723 (0.0714)	1.2699	Ovoid	Papillose (irregular)
<i>T. glomeratum</i>	0.8803 (0.0238)	0.6580 (0.0662)	1.3379	Ovoid	Obscure papillose with mounds
* <i>T. grandiflorum</i>	1.5655 (0.0547)	1.0788 (0.0366)	1.4512	Elongated-ovoid	Foveolate
* <i>T. hirtum</i>	1.8361 (0.0654)	1.3965 (0.0860)	1.3148	Ovoid	Foveolate
* <i>T. lappaceum</i>	1.1338 (0.0802)	0.9510 (0.0513)	1.1922	Round	Multi-reticulate
* <i>T. leucanthum</i>	1.6018 (0.0669)	1.1429 (1.1022)	1.4015	Elongated-ovoid	Foveolate (obscurely)
<i>T. nigrescens</i>	1.3766 (0.1055)	0.8812 (0.0621)	1.5622	Elongated-ovoid	Foveolate
<i>T. patens</i>	1.0812 (0.0294)	0.7711 (0.0581)	1.4022	Elongated-ovoid	Rugulate - reticulate
* <i>T. physodes</i>	1.7731 (1.1502)	1.5123 (0.0794)	1.1724	Round	Foveolate
* <i>T. pignantii</i>	1.9324 (0.0439)	1.5643 (0.0931)	1.2353	Ovoid	Foveolate
<i>T. repens</i>	0.8828 (0.0002)	0.7559 (0.0012)	1.1679	Heart shaped	Rugulate
<i>T. resupinatum</i> subsp. <i>resupinatum</i>	1.1339 (0.0223)	0.9162 (0.0445)	1.2376	Ovoid	Reticulate - obscurely foveolate
<i>T. stellatum</i>	2.3333 (0.1227)	1.4667 (0.1084)	1.5908	Elongated-ovoid	Papillose - rugose
<i>T. striatum</i>	1.7557 (0.0451)	1.4042 (0.0553)	1.2503	Ovoid	Papillose-rugose
<i>T. subterraneum</i>	2.2052 (0.1991)	1.9245 (0.0752)	1.1458	Heart shaped	Multi-reticulate
* <i>T. suffocatum</i>	0.8948 (0.0669)	0.7985 (0.0383)	1.1206	Round	Papillose with mounds
<i>T. tomentosum</i>	1.2204 (0.0455)	0.9417 (0.0629)	1.2960	Ovoid	Foveolate (obscurely)

and may be proved useful for delimiting taxa with dubious status at species rank that have not been examined yet. Examining more species will enable us to reassess these patterns and to describe new ones.

The general appearance of the seed surface at low magnification (43× and 150×) is tuberculate for *T. suffocatum* and *T. glomeratum* and smooth for the rest of the examined taxa. Thus, the examined species display two of the three relevant patterns found by Small & al. (1990) in the genus *Medicago* L.

There is a remarkable diversity in seed surface micromorphology of the examined species and each one has unique features making their classification a difficult task. As a result, seed surface characteristics are very suitable for the delimitation of taxa at species rank. According to the present study, seed micromorphological characters proved to be very useful for the delimitation and identification of the examined species, but generally the recognised patterns cannot support current taxonomic approaches at higher ranks. This is in agreement with the conclusions of other studies, such as Zohary & Heller (1984) and Zorić & al. (2010). However, there are some cases that infrageneric classification is supported by evidences derived from seed coat patterns. Thus, taxonomic implications of seed surface characters at ranks higher than species concern only some groups of species. Seed coat patterns cannot also support phylogenetic hypotheses nor do they reveal broader evolutionary trends as already stated by Lersten (1981).

The study will continue focusing mainly in pairs of taxa with dubious taxonomic status. Furthermore, the examination of more taxa will allow us to conduct the appropriate statistical analysis and draw broader conclusions. A more holistic approach of the study of the seed morphology and especially coat surface and its patterns will take into consideration not only its taxonomic and evolutionary significance, but its ecological-functional one too.

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