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Morphological, anatomical, pollen and seed morphological properties of *Melilotus bicolor* Boiss. & Balansa (Fabaceae) endemic to Turkey

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Abstract

The genus *Melilotus* L. has a worldwide distribution and usually grows in the calcareous soil regions of the world. It is important for its use in folk medicine, as a forage crop and for green manure. The morphology, anatomy, pollen and seed morphology of *Melilotus bicolor* Boiss. & Balansa, which is an endemic species in Turkey, were studied. An expanded and amended description of the species collected from Beypazarı to Kıbrıscık in Ankara is given. Anatomical studies were carried out on transverse sections of the roots, stems, leaves and the surface sections of the leaves. The pith of the root is completely covered by xylem cells. The stems have a chlorenchymatous cortex with many chloroplasts. The leaves are dorsiventral and amphistomatic. The layers of the mesophyll are various. Stomata are anomocytic. The pollen grains are generally trizonocolporate, radially symmetrical, isopolar and subprolate. Sculpturing is usually microreticulate or, rarely, rugulate in the meridional optical section, and microreticulate in the polar optical section. The seeds are globose to obovoid in shape, with tuberculate-rugose ornamentation.

Keywords: Fabaceae; *Melilotus bicolor*; Morphology; Anatomy; Pollen morphology; Seed morphology.
Abbreviations: LM_Light Microscope, SEM_Scanning Electron Microscope, mm_milimeter, cm_centimeter, µm_micrometer, min_minimum, max_maximum.

Introduction

According to the Flora of Turkey (Davis et al., 1988), Fabaceae is the second largest family after the Asteraceae. The genus *Melilotus* belongs to the subfamily Papilionoideae in Fabaceae under the tribe Trifolieae. It is represented by 24 species worldwide, distributed in Central and Eastern Europe, the Mediterranean countries, Asia and North America (Hoppe, 1975). In Turkey, the genus consists of 11 species and one of them, *M. bicolor*, is a taxon endemic to Turkey (Keskin, 2012) and is distributed throughout Western and Central Anatolia (Chamberlain, 1970). The category of threat to which *M. bicolor* belongs has previously been reported as LC (Least concern) (Ekim et al., 2000). *Melilotus* species, known as Melilot or Sweet-clover, are used in traditional medicines all over the world, as they possess diuretic, antirheumatic, anti-inflammatory, analgesic, antispazmodic, astringent, depressant and antiestrogenic features (Grieve, 1967; Bessin et al., 1971; Hoppe, 1975; Baytop, 1984; Rizk and Kamel, 1991; Tanker and Tanker, 1991; Tanker et al., 1992; Wagner and Wiesenauer, 1995; Anwer, 2008; Zhao et al., 2010). *Melilotus* species contain a glycoside (melilotoside), releasing glucose and coumaric acid when using the drying method. Coumarin, a secondary plant compound, is used in the cosmetic, soap and perfume sector (Yourick and Bronaugh, 1997). They also include flavanoids, resins, tannins and volatile oils (Harnischfeger and Stolze, 1983; Grigorescu et al., 1986). *Melilotus* is often used by agriculturalists as forage crops and soil builders (Turkington et al, 1978). These green manures increase the percentage of nitrogen and organic matter in the soil. Some *Melilotus* species are used for animal feed (Baytop, 1991). The family Fabaceae has been subject to a number of studies based on morphology (Baum, 1968; Ekici and Ekim, 2004; Ekici et al., 2005; Haerinasab and Rahiminejad, 2012), anatomy (Metcalfe and Chalk, 1957; Noverto et al., 1994; Mehrabian et al., 2007; Sabaii et al., 2007; Zoric et al., 2012), palynology (Clarke and Kupicha, 1976; Tewari and Nair, 1979; Ferguson and Skvarla, 1979, 1981, 1982; Diez and Ferguson 1994; Simons and Chinnappa, 2004; Pınar et al., 2009; Çeter et al., 2013) and the micromorphology of seeds (Small et al., 1989; Gupta, 1991; Estrelles et al., 2006; Salimpour et al., 2007; Vural et al., 2008; Zoric et al., 2010; Güneş and Çırpıcı, 2011; Çeter et al., 2012). There are few reports concerning the morphology, anatomy, pollen and seed morphology of *Melilotus.* The key to identifying the *Melilotus* species was provided by Schulz (1901) and Isely (1954). Gazara et al. (2001) described the morphological characters of three genera (*Trifolium* L., *Trigonella* L. and *Melilotus*) of the tribe Trifolieae in order to construct their phylogenetic relationships. In Moussavi's (2001) studies, based on the morphological characteristics of more than 200 specimens of *Melilotus* from various parts of Iran, four species are distinguished, namely *M. officinalis* (L.) Pall., *M. albus* Medik., *M. indicus* (L.) All. and *M. sulcatus* Desf.. Mcmurry and Fisk (1936) studied the vascular anatomy of *M. albus* Medik.. The vascular anatomy of the *Melilotus* flower was investigated by Gupta (1977). Perveen and Qaiser (1998) studied the pollen morphology of 157 species of the subfamily Papilionoideae in Pakistan, using light and scanning electron microscopes. They recorded that most of the tribes were easily distinguished from each other palynologically. The pollen morphology of 28 species belonging to three genera (*Melilotus*, *Trifolium* and *Trigonella*)

Table 1. Anatomical characteristics of *M. bicolor*. S.D.= standart deviation

Anatomical characters	Width (µm) Min.-Max.	Mean ± S.D.	Lenght (µm) Min.-Max.	Mean ± S.D.
Root				
Peridermis Cell	9.6-55.68	27.48 ± 11.2	8.64-20.16	13.65 ± 3.08
Cortex Cell	11.52-42.24	26.58 ± 8.79	5.76-24	13.15 ± 4.77
Trachea Cell	15.26-54.72	31.59 ± 8.26		
Stem				
Cuticle	0.72-1.92	1.02 ± 0.25		
Epidermis Cell	6.72-30.72	17.86 ± 6.57	12.48-34.56	20.08 ± 4.98
Chlorenchyma Cell	6.72-33.6	22.07 ± 6.47	8.64-29.76	16.64 ± 4.73
Trachea Cell	14.4-35.52	23.21 ± 5.7		
Pith Cell	26.88-124.8	63.17 ± 24.63		
Leaf				
Cuticle	0.72-1.44	0.92 ± 0.17		
Upper Epidermis Cell	18.24-63.36	35.4 ± 10.23	16.32-38.4	26.93 ± 5.24
Lower Epidermis Cell	14.4-54.72	35.7 ± 11.32	14.4-47.04	26.71 ± 7.69
Mesophyll Region	180-351.9	286.7 ± 34.58		
Stomata	17.28-20.16	18.6 ± 0.86	19.2-26.88	23.46 ± 2.09

Fig 1. A-B. General appearance of *M. bicolor* in field.

of the subfamily Papilionoideae in Egypt was examined by Gazar (2003) in order to determine the taxonomic position of the species within genera. Taia (2004 b) defined the pollen morphological characters of 41 species, of which four are *Melilotus*. He noticed that the variations between taxa assisted in their segregation. Lashin (2006) described the pollen grains of *M. indicus* and *M. messanensis* (L.) All. from Egypt. El-Sayed et al. (2010) investigated the pollens of 26 taxa of Papilionoideae representing 19 genera in Egypt. Thirteen seed characteristics were investigated in 30 species distributed over five genera representing the tribe Trifolieae by Taia (2004 a). It was shown that the genera *Trigonella* and *Melilotus* have variable characteristics that are used in the description of their species. So far, the morphology, anatomy, palynology and seed morphology of *M. bicolor* have not been studied. Thus, the aims of the present study are to investigate and describe the macro-micromorhological and anatomical features of *M. bicolor* and to contribute to the systematics of the genus.

Results

Morphological characteristics

Annual or biennial herbs, sparse to glabrescent. Stems erect, (10-)30-40 cm, terete, longitudinally ridged. Stipules falcate, 1.5-2 mm, entire. Leaves trifoliate; petiole slender, usually shorter than leaflets. Leaflets obovate, 6-12 x 2-7 mm, margins shallowly serrated. Racemes 1-4 cm, elongated to 8-10 cm when fruiting, 8-20-flowered; pedicels 1-1.5 mm. Corolla white, with bluish-purple tipped wings and keel, 3-6 mm. Ovary narrowly ovate with two ovules. Legume ovoid, 3-5 mm, puberulent, with longitudinal ridges. Seeds 2, dark brown to dark green in colour, globose to obovoid, papillate (Fig. 1A-B).
Diagnostic characteristics: Corolla white, with bluish-purple tipped wings and keel
Habitat: Dry rocky places, 1000-2000 m.
Flowering and fruiting time: Fl. 4-5, fr. 5-6.
Phytogeographic region: Ir-Tur. Element.
Distribution: Endemic (Wild Anatolia: Uşak, Afyonkarahisar, Ankara).
Threat category: LC

Anatomical characteristics

Root: The transverse sections of the roots reveal that the periderm is 3-6 layers on the outermost surface and its cells are squashed or breaking up. Under the periderm, the parenchymatic cortex has 4-9 layers. A few sclerenchyma cells come together and create small bundles. The cambium is not distinguishable. The xylem rays are composed of 1-3 rows of cells. The pith is occupied by the xylem (Fig. 2A-B).
Stem: The transverse section of the stem, the epidermis, is covered by a thin layer of cuticle. There are eglandular and glandular hairs on the epidermis. The epidermis consists of uniseriate, differently sized and shaped cells. The lateral walls of the epidermis cells are thinner than the upper and lower walls. The collenchyma tissue, which is located just under the epidermis, is 3-4 layered at the corners and is single-layered between the corners. There are 3-4 layers of chlorenchymatous cortex with many chloroplasts below the collenchyma. A single layered endodermis is located above the vascular bundles. The sclerenchymatical ring above the phloem is 2-4 layered. The cambium is distinguishable. The xylem elements have a larger area than the phloem. The pith is wide and consists of polygonal or orbicular parenchymatous

Table 2. Pollen morphological parameters of *M. bicolor* (values in µm).

P		E	P / E ratio	Colpus (Cl)	
				Clg	Clt
30.72-35.52 32.04 ± 1.12		24-26.64 25.12 ± 0.77	1.18-1.4 1.27 ± 0.05	24-26.88 25.72 ± 0.88	4.08-4.8 4.59 ± 0.27
Pore (Pl)		Exine	Intine	Amb	t
Plg	Plt				
7.2-8.64 7.77 ± 0.28	8.16-9.6 8.98 ± 0.45	0.48-0.96 0.73 ± 0.1	0.24-0.48 0.46 ± 0.06	24-25.92 24.92 ± 0.53	15.36-19.2 16.86 ± 1.45

P: Polar axis; E: Equatorial axis; Clg: Colpus lenght; Clt: Colpus width; Plg: Pore length; Plt: Pore width; Amb: Equatorial outline, t: Diameter of apocolpium; data are the range, with the mean and standard deviation.

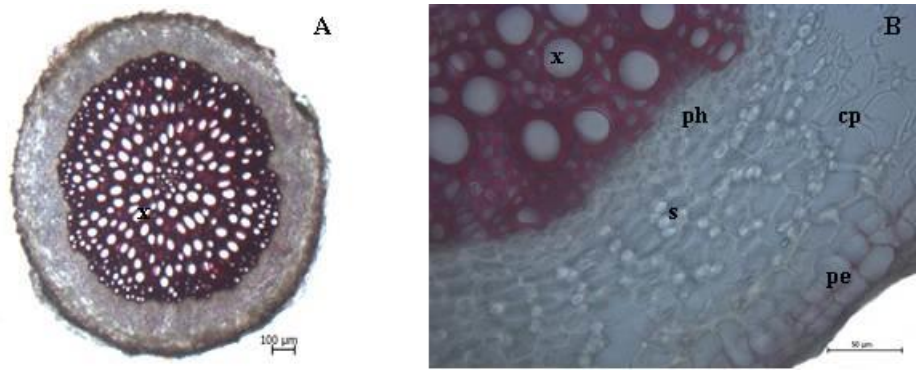

Fig 2. The transverse sections of the root of *M. bicolor*. A- General view, B- Enlargement showing internal structure, cp: cortex parenchyma, pe: periderm, ph: floem, s: sclerenchyma, x: xylem.

cells (Fig. 3A-C). Leaf: The transverse sections of the lamina and the midrib of *M. bicolor* show that the upper and lower epidermises are covered with a thin cuticle layer. The thickness of the cuticle of both epidermises is nearly equal. There are densely eglandular and sparsely glandular hairs on the lower epidermis only. Both epidermises consist of uniseriate cells with different sizes and shapes. The leaf is dorsiventral (bifacial) and amphistomatic. The mesophyll is composed of 2-3 layers of elongated, rectangular palisade parenchyma cells and 3-4 layers of spongy parenchyma cells. There is a large vascular bundle on the median region of the leaf. Small bundles are embedded or vertically transcurrent in the mesophyll. Vascular bundles are surrounded by parenchymatic cells (Fig. 4A-B). In the surface sections, epidermis cells are longitudinally elongated and have wavy walls. Stomata are anomocytic (Ranunculaceous type) and the guard cells are surrounded by 3, 4 or rarely 5 subsidiary cells. They are found on both surfaces, but the upper epidermis has fewer stomata (Fig. 5A-B, Table 1).

Pollen characteristics

The pollen grains of *M. bicolor* are 98% trizonocolporate, 2% syncolporate and are isopolar and radially symmetrical. Their shape is subprolate. The polar axis (P) is 32.04 ± 1.12 µm and the equatorial axis is (E) 25.12 ± 0.77 µm. The ratio of P/E is 1.27 ± 0.05 µm. The colpi are long and narrow with clear margins (Clg 25.72 ± 0.88 µm, Clt 4.59 ± 0.27 µm). Pores are lalongate or circular (Plg 7.77 ± 0.28 µm, Plt 8.98 ± 0.45 µm). The aperture membrane is granulate. The amb is 24.92 ± 0.53 µm and subcircular. The exine thickness is 0.73 ± 0.1 µm. The intine thickness is 0.46 ± 0.06 µm. The diameter of the apocolpium is 16.86 ± 1.45 µm (Fig. 6A-E). The exine sculpturing is 90% microreticulate and 10% rugulate in the meridional and microreticulate in the polar optical sections (Fig. 7A-C, Table 2).

Seed morphology

The seeds are dark brown to dark green in colour and are globose to obovoid in shape. The size of the seeds is 1.65 ± 0.12 mm in width and 2.19 ± 0.07 mm in length. The hilum is elliptic (Fig. 8A). The seed coat ornamentation is tuberculate-rugose (Fig. 8B).

Discussion

The present study provides useful information regarding the morphology, anatomy, palynology and seed morphology of *M. bicolor*. This is the first report on these characteristics of *M. bicolor*. It differs morphologically from the other species of *Melilotus* in that it has white flowers with bluish-purple-tipped wings and keel. Although the present results usually correspond to the description recorded in Flora of Turkey (Chamberlain, 1970), there are several differences concerning with the description in the Flora of Turkey: the stem is (10-) 30-40 cm (not 10-15 cm), the corolla is 3-6 mm (not 5-6 mm) and the legume is 3-5 mm (not c. 4.5 mm). Some additional morphological characteristics of *M. bicolor* are also given in Table 3. Metcalfe and Chalk (1957) provided important information regarding the anatomy of the subfamily Papilionaceae. They pointed out that the pith rays of the roots of this subfamily are composed of 1-12 (mostly 2-3) or more-rowed cells. The present study of cross sections of the root of *M. bicolor* shows that this species has 1-3 rows of ray cells. The hairs are glandular and non-glandular. The non-glandular hairs are uniseriate, with a variable number of

Table 3. Comparison of morphological characters of *M. bicolor* based on our study and the Flora of Turkey.

Characters	Our measurements	Flora of Turkey
Habit	annual or biennial	-
Indumentum	sparse to glabrescent	-
Stem	(10-)30-40 cm	10-15 cm
Leaflets	6-12 x 2-7 mm	-
Stipules	1.5-2 mm	-
Pedicels	1-1.5 mm	-
Corolla	3-6 mm	5-6 mm
Legume	3-5 mm	c. 4.5 mm
Ovary	narrowly ovate	-
Seed	globose to obovoid	-

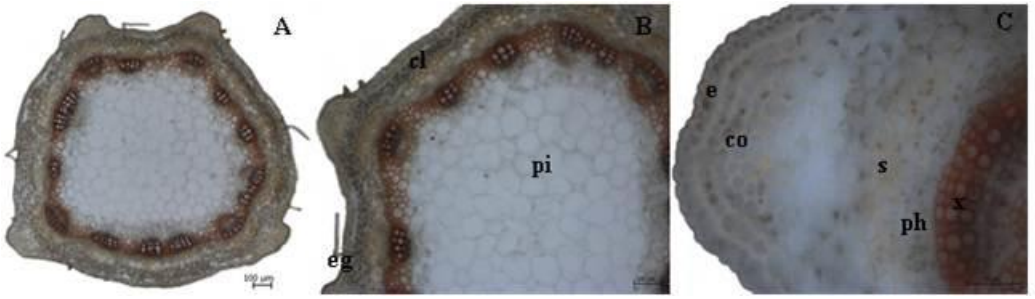

Fig 3. The transverse sections of stem of *M. bicolor*. A- General view, B- Enlargement showing internal structure, C- The corner, cl: chlorenchyma, co: collenchyma, e: epidermis, eg: eglandular hair, ph: phloem, pi: pith, s: sclerenchyma, x: xylem

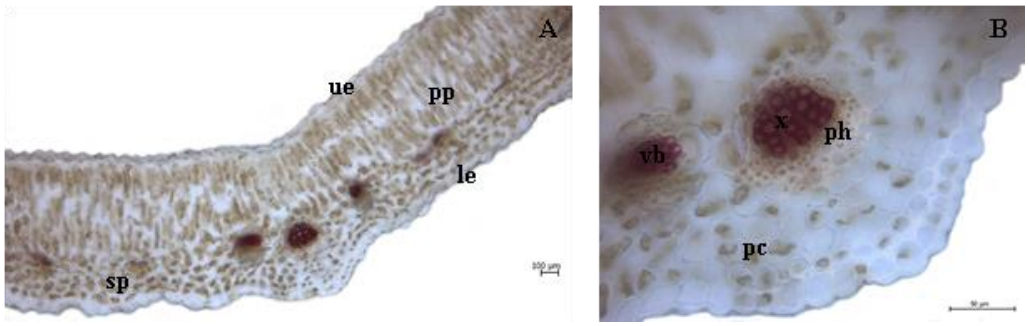

Fig 4. The transverse sections of leaf of *M. bicolor*. A. General view, B. The midrib, le: lower epidermis, pc: parenchymatous cell, ph: floem, pp: palisade parenchyma, sp: spongy parenchyma, ue: upper epidermis, vb: vascular bundle, x: xylem.

short basal cells, accompanied by an elongated terminal cell. In glandular types, they are club-shaped, with or without a distinct stalk in *Melilotus*. The anticlinal walls of the leaf epidermis are provided with angular folds in certain species like *Melilotus*. Stomata are present on both surfaces in all investigated Trifolieae. The leaf is very variable in structure because of the wide range of leaf types in the family, although it is usually dorsiventral. Smaller veins are embedded or vertically transcurrent, with the latter type being particularly recorded in certain Trifolieae (Metcalfe and Chalk, 1957). We observed the same anatomical properties in the leaf of *M. bicolor*. The pollen grains of *M. bicolor* are trizonocolporate, isopolar and radially symmetrical. Their shape is subprolate. The exine sculpturing is mostly microreticulate. The pollen in *M. indicus* is tricolporate, subprolate-prolate and the sculpture is microreticulate. *M. messanensis* were observed to be subprolate and the sculpturing to be microreticulate-perforate. The morphological characteristics of the pollen were considered diagnostic at the generic and specific level of studied Papilionoideae (El-Sayed et. al., 2010). Pollen grains in *M. albus*, *M. sulcatus*, *M. siculus* (Turra) B.D. Jackson and *M. indicus* are perprolate, rarely subprolate, tricolpate with long, narrow and grooved colpi with smooth membranes and ornamented margins. The exine is reticulate (Taia, 2004 b).

The seed-coat pattern, or the micro-ornamentation on the surface of the outer cell wall, can be considered to be of high taxonomic value in the identification of the species, as indicated by Barthlott and Frolic (1983), Berchtold and Presol (1820) and Taia (2004 a). The seeds of *M. bicolor* are globose to obovoid in shape and are 1.65 ± 0.12 x 2.19 ± 0.07 mm in size. The surface ornamentation is tuberculate-rugose. The seeds of *M. albus*, *M. sulcatus*, *M. siculus*, *M. elegans* Ser. and *M. indicus* have been reported to observe great variations in seed shapes and testa ornamentations. While the seed shapes of *M. sulcatus*, *M. elegans* and *M. indicus* are

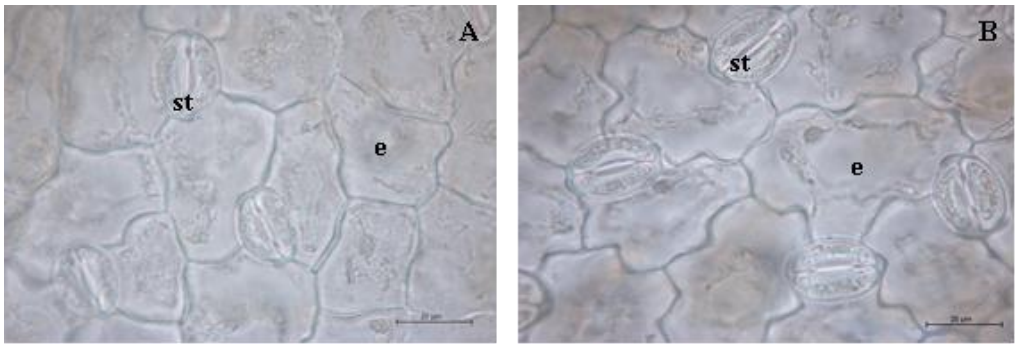

Fig 5. The surface sections of leaf of *M. bicolor*. A. The stomata from upper epidermis of leaf, B. The stomata from lower epidermis of leaf, e: epidermis, st: stoma.

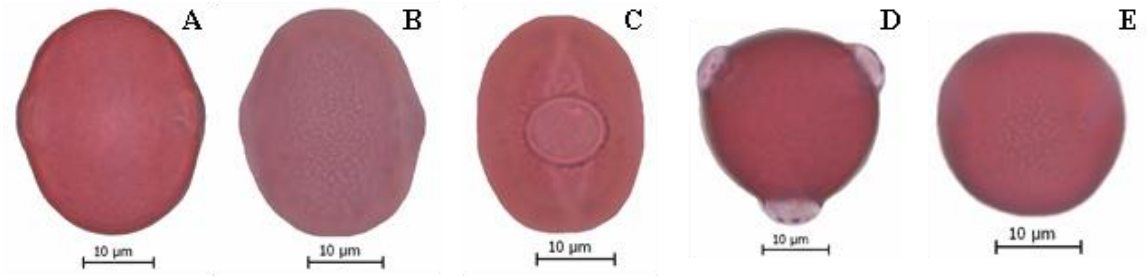

Fig 6. LM micrographs of the pollen of *M. bicolor*. A. Equatorial view, B. Ornamentation (Equatorial), C. Colpus and pore, D. Polar view, E. Ornamentation (Polar).

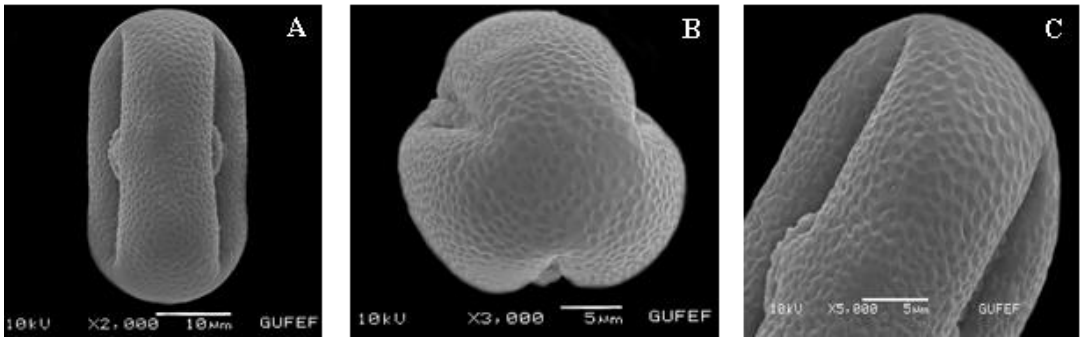

Fig 7. SEM micrographs of the pollen of *M. bicolor*. A. Equatorial view, B. Polar view, C. Exine ornamentation.

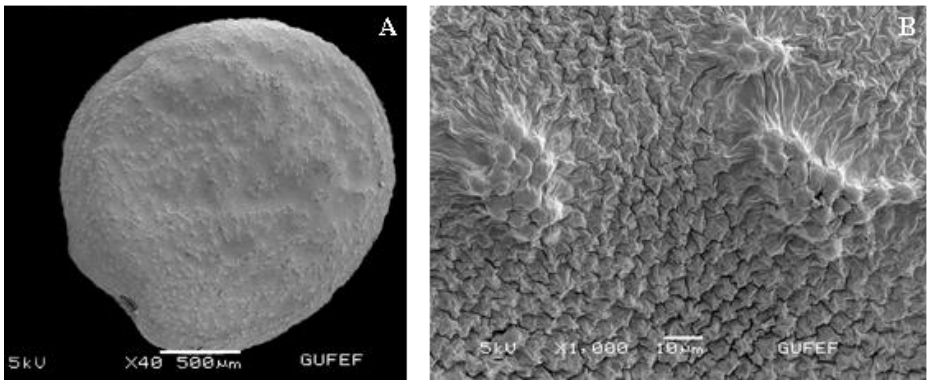

Fig 8. SEM micrographs of seed of *M. bicolor*. A. General view, B. Testa ornamentation.

oval, in *M. albus* they are lenticular. The seed-surface ornamentation is smooth in *M. albus*, papillate in *M. sulcatus*, granulate in *M. elegans* and striate in *M. indicus* (Taia, 2004 a).

Material and Methods

Plant materials

The specimens were collected during a field trip in Ankara (A3 Ankara: 15 km from Beypazarı to Kıbrıscık, edge of *Quercus* forests, slopes, 1000 m, 11.05.2012, *U. Özbek* 2850 & *M. Ekici*). The voucher specimens have been deposited at the Gazi University Herbarium (GAZI).

Anatomical investigations

Anatomical studies were carried out on specimens fixed in 70% alcohol. The cross sections of the roots, stems and leaves were done by hand and were stained with Floruoglycine-HCl. At least 30 measurements for each parameter were performed using an ocular micrometer on a light microscope. Slides were photographed using a Leica DM1000 binocular light microscope with the Leica DSCF3200 camera.

Palynological investigations

Pollen materials were obtained from the herbarium samples for the palynological investigations. Pollen slides were prepared using the technique of Wodehouse (1935). For LM studies, measurements were taken from 20 or more pollen grains for each morphological characteristic using an ocular micrometer on a light microscope. Photographs were made using the Leica DM1000 binocular light microscope with the Leica DSCF3200 camera. For SEM, dry pollen grains were mounted on stubs and were covered with gold. They were observed and photographed with a JEOL JSM 6060 scanning electron microscope. The pollen terminology of Faegri and Iversen (1992) and Punt et al. (1994, 2007) were used. The class of pollen shape, based partly on P/E ratio, was identified using Erdtman's (1969) system.

Seed morphological investigations

The seeds were first investigated using a Leica EZ4D stereomicroscope. In order to determine the average sizes, 20 mature seeds were measured. For SEM, the mature seeds were placed on stubs and were coated with gold. They were examined and photographed with a JEOL JSM 6060 scanning electron microscope.

Conclusion

In this study, the morphology, anatomy, pollen and seed morphology of *M. bicolor* were determined for the first time. We think that our results will contribute to systematics of this genus when studying other species of *Melilotus*.

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