



Taxonomic revision of *Melilotus indicus* complex in Egypt

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Abstract

Macro- and micro-morphological features of *Melilotus indicus* complex in Egypt were examined using light and scanning electron microscopy, these include embryo and seed characters, anatomical structure of stem and seed spermoderm. In the present study, three varieties were recorded: var. *indicus*, var. *bonplandii* and var. *tommasinii*, of which var. *indicus* is a new record to the flora of Egypt. The study revealed that morphological, anatomical and seed characters are of taxonomic significance. A key for identification of the varieties was also given.

Keywords: anatomy, *Melilotus indicus*, morphology, seed morphology, spermoderm

Introduction

Fabaceae Lindl. is the third largest family of flowering plants with about 751 genera and 19500 species (Christenhusz and Byng 2016), distributed mainly in the temperate and subtropical parts of the world. Many of the species are of economic importance as the source of food, fixation of atmospheric nitrogen etc. (Mirzaei *et al.* 2015). The genus *Melilotus* L. (subfamily Papilionoideae, tribe Trifolieae), is represented by 24 species with a worldwide distribution growing usually in the calcareous soil regions distributed in Central and Eastern Europe, the Mediterranean countries, Asia and North America (Hoppe 1975).

There are few studies concerning the morphology, anatomy, pollen and seed morphology of *Melilotus*. Mcmurry & Fisk (1936) studied the vascular anatomy of *M. albus*. Özbek *et al.* (2014) investigated morphological, anatomical, pollen and seed morphological properties of *Melilotus bicolor* endemic to Turkey; while Gupta (1977) investigated the vascular anatomy of the *Melilotus* flower. Abid *et al.* (2018) investigated seed characters and chemotaxonomy of four species of *Melilotus* from Pakistan including *M. indicus*. Gazara *et al.* (2001) described the morphological characters of three genera: *Trifolium* L., *Trigonella* L. and *Melilotus* L. of the tribe Trifolieae in order to construct their phylogenetic relationships. Taia (2004) studied seed characters in 38 species of tribe Trifolieae, which showed that *Melilotus* have

different seed shape and ornamentations, which can be used in the identification of the species. Lashin (2006) investigated the pollen grains of nine taxa belonging to five genera of the tribe Trifolieae of subfamily Papilionoideae from Egypt including *Melilotus indicus* to evaluate the comparative morphology of the pollen in the tribe. The seedling of Leguminosae was discussed by Duke & Polhill (1981), they claimed that the combination of seed and adult features represented on seedlings may provide numerous clues for field. Various works have shown that the microcharacters of seed coat, anatomical features and pollen grain features in Leguminosae can be used as diagnostic characters at generic, specific and even at infraspecific levels. Seed morphology and structure in the Leguminosae has been the base of numerous studies (Gunn, 1972; Ignacimuthu & Babu, 1985; Abd El-Gawad *et al.* (1989); Hosni & Karemy 1993, Abou-El-Enain & Loutfy 1999, El Karemy & Hosni 1999, Abou-El-Enain *et al.* 2007; Nyola & Sharma, 2009; Özbek *et al.* 2014; Zareh *et al.* 2017; Khandani *et al.* 2016; Abid *et al.* 2018)

In Egypt *Melilotus* is represented by seven species (El-Hadidi & Fayed 1994/95; Boulos 1995, 1999, 2009), while Täckholm (1974) recorded only six species of *Melilotus*. She recorded two varieties within the multiform species *M. indicus* namely: var. *tommasinii* Jord. and var. *bonplandii* Ten. The present study aims to revise *Melilotus indicus* complex in Egypt with special reference to

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macromorphological and anatomical features as well as embryo and seed structure that hoped to be reliable for the distinction of different varieties; described their distribution and habitat in Egypt along with its morphological, palynology, seed, embryo and anatomical characters.

Materials and Methods

The present study based on fresh materials collected from various regions in Egypt, in addition to herbarium specimens deposited in Menoufia University Herbarium (Acronym MNF). The specimens were identified using various books of Flora (Täckholm 1974, Boulos 1999).

For Macromorphological Studies of embryo: mature seeds were soaked in tap water over night; testa was removed then examined using stereomicroscope then photographed using Zeiss research microscope.

For the Anatomical Studies: samples for anatomy of stem were chosen from both herbarium and fresh material. All assessments were made on all plants at similar developmental stages (fruiting stages) and in comparable positions of each plant. Stem samples were taken from 4th internodes from the apex about 2-3 cm. Fresh materials were fixed in F.A.A. (5:5:90), while dried herbarium specimens of stem and leaves were first softened by either normal or warm water, after fixation stem and leaves specimens were transformed in ethyl alcohol series, then embedded in paraffin wax. The specimens were sectioned at 10-15 μm ; sections were dehydrated in alcohol-xylol series. Sections were stained in safranin and light green according to Sass (1961). The transverse sections of stem and leaves were examined by using light microscope. A Planimeter was used for estimation of each tissue in the section area. Terminology followed Abd El-Rahman *et al.* (1976); Pandey (1982) and Barthlott (1981).

For the study of seeds: at least 20 seeds from different individuals were examined by Light microscope (LM) to assess morphological characters and general features of the spermoderm. For SEM microscopy, seeds were mounted on brass stubs and coated with a thin layer of gold and examined using JEOL JSM 530P SEM at Electron Microscopic Unit, Faculty of Science, Alexandria University. For anatomical investigation of seeds, the seeds were soaked in water for 12 hrs, embedded in paraffin wax, microtomed at 12-15 μm , dehydrated and stained with safranin according to conventional method of Johansen (1940). Terminology followed Corner (1976), Lersten (1981), Barthlott (1981, 1984), Stearn (1992), Werker (1997) and Norbert & Jonitz (2009).

The results were summarized in Tables (1-4) showing the variation between the studied varieties. Photographed obtained were shown in Figures (I-IV).

Results and Discussion

According to Schulz (1901), *Melilotus indicus* has three varieties, var. *indicus*, var. *tommasinii* and var. *bonplandii*; while Morton (1915) recorded only two varieties: var. *indicus* and var. *tommasinii*. Täckholm (1974) reported two varieties from Egypt: var. *tommasinii* and var. *bonplandii* based on plant size, number of flowers, legume length while El Hadidi & Fayed (1994/95) and Boulos (1999, 2000 & 2009) recorded the species without infraspecific taxa. No comprehensive studies on *Melilotus indicus* in Egypt had been done before, only few studies were done among the revision of some other taxa (e.g. Taia 2004, Gazara 2001, Lashin 2006). In the present study three varieties were recognized within *M. indicus* based on Macromorphological characters (habit of stem, hairs and midrib colour, flower characters) as well as embryo characters; radical shape, anatomical features of stem (epidermal and collenchyma cell shape, number of vascular bundles) and seed coat pattern (curvature of outer periclinal wall) which confirmed the

morphological results to great extent and agreed with Schulz's concept (1901). Our results, however, revealed that such characteristics can play a determining role in differentiating between different

varieties and distinguishing them from one another. The varieties of *M. indicus* in Egypt can be distinguished according to the following key:

- 1a-Plant erect, radical in embryo cylindrical, collenchyma cell shape radially elongated, seed coat pattern rugose-tuberculate in between.....**var. indicus**
 1b-Plant procumbent, radical in embryo ovate, collenchyma cell shape isodiametric-tangentially elongated, seed coat pattern verrucate with irregular surface 2
 2a- Leaves with red vein (midrib), number of flowers up to 25, standard with obtuse apex, ovary elliptic, number of vascular bundles 13-14, Curvature of outer periclinal wall convex**var. bonplandii**
 2b- Leaves with colorless vein (midrib), number of flowers 30-35, standard with emarginated apex, ovary oblong, number of vascular bundles 18-20, curvature of outer periclinal wall concave..... **var. tommasinii**

Taxonomy and morphological studies
(Table 1).

Melilotus indicus (L.) All. Fl. Pedem. 1:308(1785) var. *indicus*.

Basionym: *Trifolium melilotus-indica* L.Sp.Pl.ed.1:765(1753).

Annual herb, up to 30cm height, stem erect, hairy, branched from base, lower branches cylindrical with protrudes, upper branches angular, internodes 2-3.5 cm long. Leaves 2.5-4(5) cm long, petiolate, petiole 0.8-1.5(2) cm long, lamina 12-18(20) x4-6mm, elliptic, sparsely hairy, serrate-ciliate margins, and acute apex. Stipules 6-8x2-3mm, ovate, entire margin, acute-acuminate apex. Inflorescence raceme, 1.2-1.5 cm long, shorter than the bract, 25-30 flower; flowers sessile-subsessile, 1.8-2 mm long; calyx hairy, 1.2-1.5 mm long, connate with teeth, tube 0.8-1 mm long, longer than teeth, teeth 0.4-0.5 mm long; petals yellow, standard 1.8-2x0.8-1 mm, longer than wings and keels, standards lamina elliptic with emarginate apex; wings as long as keels or slightly shorter, wings 1.6-1.8 mm long with obtuse apex, lamina 1-1.2 mm long, claw 0.4-0.6 mm long; keels 1.8-2 mm long with acute-obtuse apex, lamina 1.4-1.6 mm long, claw 0.4-0.6 mm long. Stamens diadelphous, 1.6-1.8 mm long, free filaments 0.8-1 mm long, anther ovate-oblong, 0.6x0.8

mm long. Carpel one, 1.6-1.8 mm long, ovary 0.8-1 mm long, ovoid, papillate, style 0.6-0.8 mm long, stigma up to 0.2 mm long, ovate. Pod globose with reticulate ridges and acute apex, 3-3.24x1.8-2mm, indehiscent, 1-2 seeded. Seeds ovoid, yellow-brown.

Melilotus indicus (L.) All. var. *bonplandii* (Ten.) O. E. Schulz, Bot. Jahrb. Syst. 29:715. (1901)

Basionym: *Melilotus bonplandii* Ten., Index Sem. Hort. Neapol. 1833:14(1833)

M. indicus var. *bonplandii* differ from var. *indicus* in stem being cylindrical in lower part and cylindrical with ridges in upper part. Flowers 30-35. Calyx 1.8-2 mm long, petal 2.8-3.5 mm long. Stamens up to 2.5 mm long. Carpel 1.8-2.8 mm long.

Melilotus indicus (L.) All. var. *tommasinii* (Jord.)O. E. Schulz, Bot. Jahrb. Syst. 29:714. (1901).

Basionym: *Melilotus tommasinii* Jord., Mém. Acad. Roy. Sci. Lyon. Sect. Sci., ser.2, 1: 266 (1851)

M. indicus var. *tommasinii* differ from var. *indicus* in stem by having up to 50 cm, stem cylindrical in lower part and cylindrical with ridges in upper part, Leaves up to 3 cm long. Leaflets with red veins, entire-dentate margins. Calyx 1.4-1.6 mm long, corolla 3 mm long; androecium up to 2.5 mm long; gynoecium 1.8-2.8 mm long, ovary elliptic.

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Table (1): Comparison of morphological characters of *Melilotus indicus* varieties.

Characters/ Taxa		<i>var. indicus</i>	<i>var. bonplandii</i>	<i>var. tommasinii</i>	
Habit		Annual--erect	Annual-procumbent,	Annual-procumbent	
Length		Up to 30 cm	Up to 20 cm	Up to 50 cm	
Stem outline		Cylindrical with ridges	Cylindrical	Lower cylindrical upper with ridges	
Leaves	Length	2.5-5cm	2-3 cm	2-3 cm	
	Leaflets	Shape	Elliptic with green veins	Obovate elliptic with red veins	Elliptic with green veins
		Margin	Serrate-ciliate	Entire – dentate	Serrate-ciliate
	Apex	Truncated -acute ape	Truncate	Truncate - acute	
	Hairs	Sparsely hairy	Dorsal side more hairy than anterior one	Sparsely hairy with tufts at lamina base	
Stipule	Shape	Ovate	Ovate	Ovate	
	Apex	Acuminate	Acute	Acute-acuminate	
	Hairness	Sparsely hairy	Sparsely with tufts inside	Sparsely hairy	
No of flowers/ inflorescence		20-25	22-25	30-35	
Calyx	Length	2-2.2 mm	Up to 3 mm l.	3-3.5 mm	
	Tube	0.9-1.1 mm	0.8-1 mm	0.9-1 mm	
	Teeth	0.2-0.3 mm	0.4-0.6 mm	0.9-1 mm	
Corolla	Colour		Yellow	yellow	Yellow
	Standard	Shape	Elliptic	Elliptic	Elliptic
		Length	2-2.2 mm	Up to 3 mm	2.5-3.5 mm
		Apex	Emarginate	Obtuse	Emarginate
	Wings	Length	1.8-2 mm	2.5-2.8 mm	3.2-3.5 mm
	Keel	Length	1.8-2 mm	2.6-2.8 mm	3-3.2 mm
Androecium	Filament	0.9-1 mm	1.2-1.5 mm	1.5-1.7 mm	
	Anther	0.6 mm	0.8-1 mm	0.7-0.9 mm	
Gynoecium	Ovary	Length	0.6-1.1 mm l.	1.5-1.8 mm	
		Shape	Oblong	Ellipsoid	Oblong-papillae
	Style+ stigma	1 mm	1-1.4 mm	1 mm	
Pod	Colour	Brown	Brown	Brown	
	Shape	Obovate with reticulate ridges	Obovate with reticulate ridges	Obovate with reticulate ridges	
	Diameter	3.2x1.8 mm	3-3.5x2 mm	3.5-4x2.5 mm	
Seed	Diameter	1.8-2x1.5 mm	2-2.5x1.5 mm	2.5-3.5 x2.2mm	
	Colour	Brown	Brown	Brown	

Embryo studies: (Fig.1)

Cotyledons yellow to pale brown in the studied varieties except in var. *indicus* (Fig.1a); it is brown, cotyledons oblong,

radical accumbant, cylindrical in var. *indicus*, conical in the other two varieties; radical with mucilage attached to testa in the three studied varieties

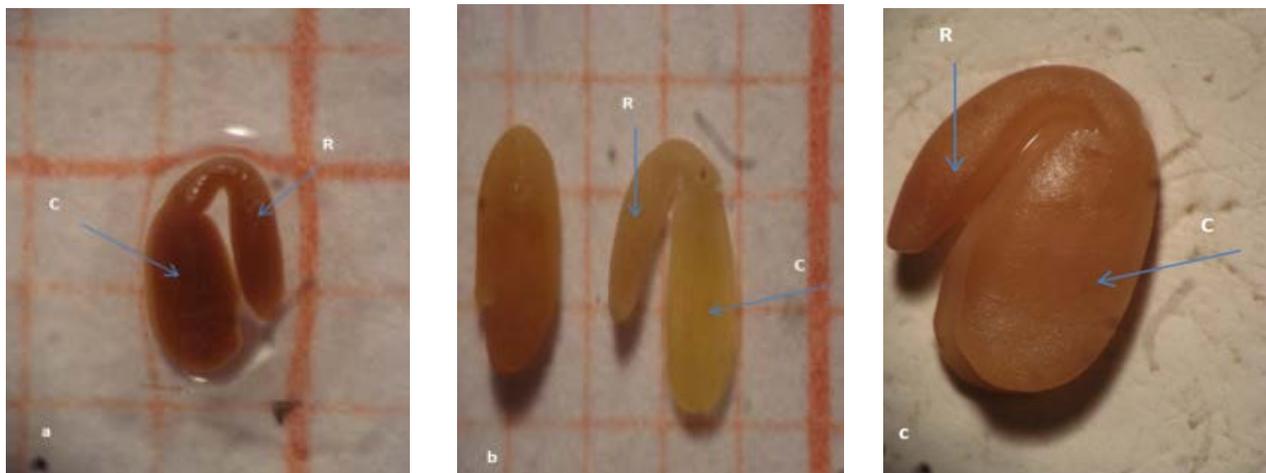


Figure I: Cotyledons and radical shape and colour of *Melilotus indicus* varieties: a. var. *indicus*, b. var. *bonplandii*, b. var. *tommasinii*. ; R. Radicle, C. Cotyledons.

Anatomical studies: (Table 2, Fig. II)

Stuessy (1990) stated that the anatomical data have been used at all levels of taxonomic hierarchy as well as for identification and assessment of taxonomic relationships among the taxa of flowering plants.

Zaki *et al.* (1991) proved that the characters of the cortical and vascular cylinder in stem anatomy provide a reliable character for distinction of the different species. In the present study, the outline of stem is polygonal in all the studied varieties. Epidermal cells were radially elongated in var. *indicus* (Fig. II, a,b) tangentially elongated in var. *bonplandii* (Fig. IIc,d) and tangentially elongated-radially in var. *tommasinii*, (Fig. II ,e,f); cell width were 15-20 (30) μm in all the studied varieties. Cortex consists of collenchyma followed by parenchyma. Collenchyma represented by 2-3 layers, radially elongated in var. *indicus*, 2-3 layers, tangentially elongated var. *bonplandii* and 1-2 layers at angles only, isodiametric-tangentially elongated in var. *tommasinii*. The

number of collenchyma layers were found useful in the delimitation of the studied taxa , this result coincide with the study of Shaheen (2007) who stated that the number of collenchyma layers and position, which is of taxonomic importance among the studied species of Caesalpinoideae (Fabaceae). Parenchyma 2-4 layers, tangentially elongated in all varieties. Phloem fiber is in the form of sclerenchymatous patches facing vascular bundles; (2) 4-6 layers in all the studied varieties. Bundle sheath well defined, tangentially elongated cells; a layer of tubular cells circles the bundle. The number of vascular bundles was found significance in the studied taxa; it was 18-20 in var. *indicus* and var. *bonplandii* while 13-14 in var. *tommasinii*. Phloem up to 4 layers, 12-18 μm wide, cambium 1-2 layers, up to 5 μm wide, Xylem arches 3-6 (7), vessels 4-7 (8) per arch in all the studied varieties. Pith cells isodiametric in var. *indicus*, isodiametric - tangentially elongated in var. *bonplandii* and was partially hollow in var. *tommasinii*.

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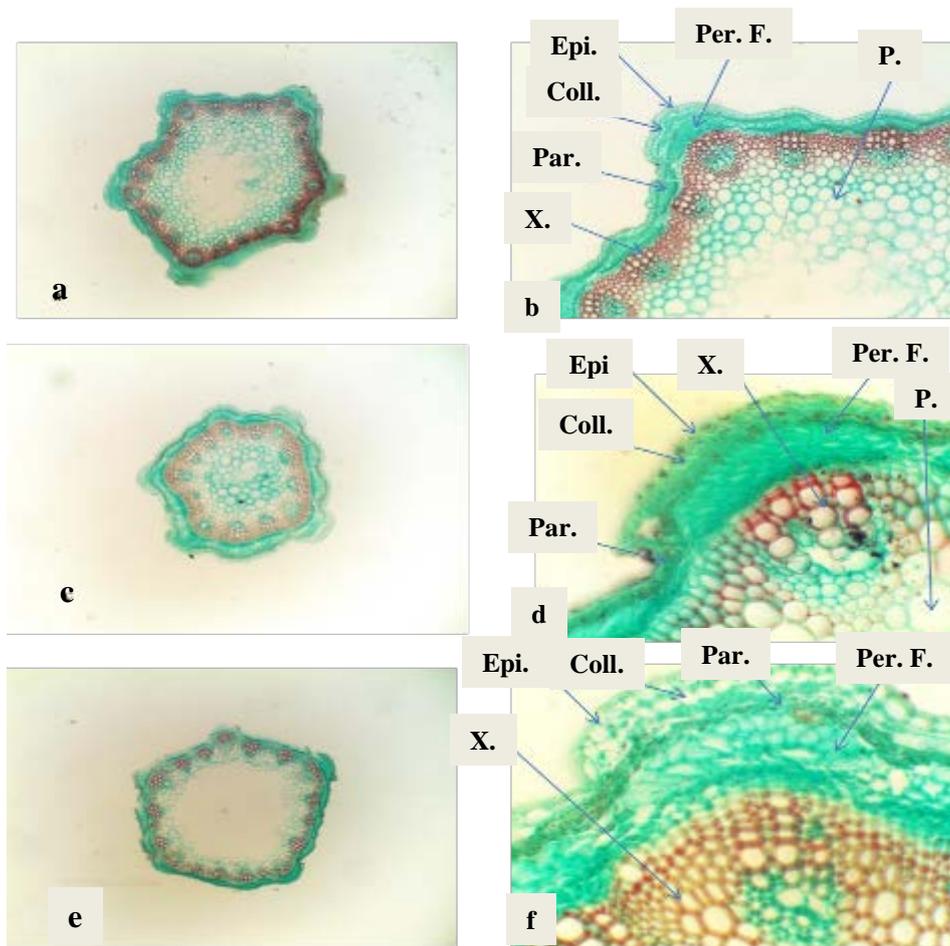


Figure II. Anatomical studies of stems of *Melilotus indicus*: a, b, var. *indicus*; c, d, var. *bonplandii*; e, f, var. *tommasinii*. Epi.; epidermis, Coll.; collenchyma, Par.; Parenchyma, Per. F.; pericyclic fibers, X.; xylem., P; pith

Table (2): Anatomical characters of stem in *Melilotus indicus* varieties.

Characters		var. <i>indicus</i>	var. <i>bonplandii</i>	var. <i>tommasinii</i>
Outline		Polygonal(5-6-ogonal)	Pentagonal	Pentagonal
Diameter mm		1.6-1.7	1.4-1.6	1.2-1.3
Epidermal cell	Shape	Radially elongated	Tangentially elongated	Tangentially elongated-radially elongated at angles
	Width μm	15-20	20-30	17-20
Cortex	Collenchyma	Number of layers	2-3	2-3
		Cell shape	Radially elongated	Tangentially elongated
		Width μm	35-50	30-40
	Parenchyma	Number of layers	3-4	2-3
		Cell shape	Tangentially elongated	Tangentially elongated
	Width μm	40-55	12-20	
Vascular bundles		Number	18-20	13-14
	Width μm	260-270	150-165	170-185
phloem fiber	Number of layers	4-6	2-4	4-5
	Width μm	25-30	20-30	40-50
Phloem	Number of layers	3-4	2-3	3-4
	Width μm	12-18	5-10	20-25
Cambium	Number of layers	1-2	1-2	1-2
	Width μm	2-4	2-5	3-5
Xylem	Number of arches	3-6	3-7	3-5
	Number of vessels	4-7	3-6	3-8
	Width μm	160-175	100-115	100-120
Pith	Cell shape	Isodiametric	Isodiametric-radially elongated	Isodiametric
	Diameter μm	750-775	900-950	500-600

Seed studies (Table 3, Fig. III)

1. Seed Morphology and Spermoderm

Many of the seed identifications have recently been based upon morphological characteristics including seed size, general appearance, outer shape and hilum length etc.; seeds of family Fabaceae were much useful for classification and of taxa at different levels (Gunn. 1981; Capitaine 1912). Seed coat pattern or the micro ornamentation on the surface of the outer cell wall can be considered of taxonomic significance (Barthlott 1981, Gupta 1991, Small *et al.*1990, Husain *et al.* 1994, Abou Sbaih *et al.*1994, Taia 2004, Salimpour *et al.* 2007, Pinaretal.2009, Abid *et al.* 2018).

In the present study the general shape of the seeds in the three taxa were prolate-

spheriodal with rounded poles, brown with longitudinally subapical hilum (Fig.3, c,h,m). Seed length 1-1.4 µm in the three varieties. Length/width ratio up to 1-1.3 in both var. *indicus* and var. *tommasinii* (Fig.3, k) while it was up to 1.87 in var. *bonplandii* (Fig.3f). Seed coat pattern rugose-tuberculate in var. *indicus* (Fig.3e), verrucate with irregular surface in the other two varieties. Outline of cells polygonal, isodiametric-irregular in the studied taxa. Anticlinal wall lobbed in var. *indicus* and var. *tommasinii* while Sinuate in var. *bonplandii*. Relief of cell boundary raised in var. *indicus*, channeled in in the other varieties. Curvature of outer periclinal wall convex in var. *indicus* and var. *bonplandii* while concave in var. *tommasinii* (Fig. 3, e,j,o respectively).

Table (3): Comparison of size, shape, spermoderm and hilum features in examined *Melilotus indicus* varieties.

Character\Species		var. <i>indicus</i>	var. <i>bonplandii</i>	var. <i>tommasinii</i>
Seed size	*LXW mm	0.93 x 0.7	1.367x0.7	1.07x0.933
	*L\W ratio	1.3	1.87	1.14
Seed poles		Rounded	Rounded	Rounded
Seed shape		Subprolate	Prolate	Prolate-spheriodal
Seed coat pattern		Rugose-tuberculate	Verrucate with irregular surface	Verrucate with irregular surface
Hilum	Position	Subapical	Subapical	Subapical
	Shape	Longitudinally	Longitudinally	Longitudinally
		Elliptic	Elliptic	Elliptic
Size µm	68x68	68x73	68x73	
Outline of cells		Polygonal, elongated-irregular	Polygonal	Isodiametric-polygonal
Anticlinal wall		Lobbed	Sinuate	Lobbed
Relief of cell boundary		Raised	Channeled	Channeled
Thickness of cell boundary		Thick	Thick	Moderate
Curvature of outer periclinal wall		Convex	Convex	Concave

*L; length

*W; width

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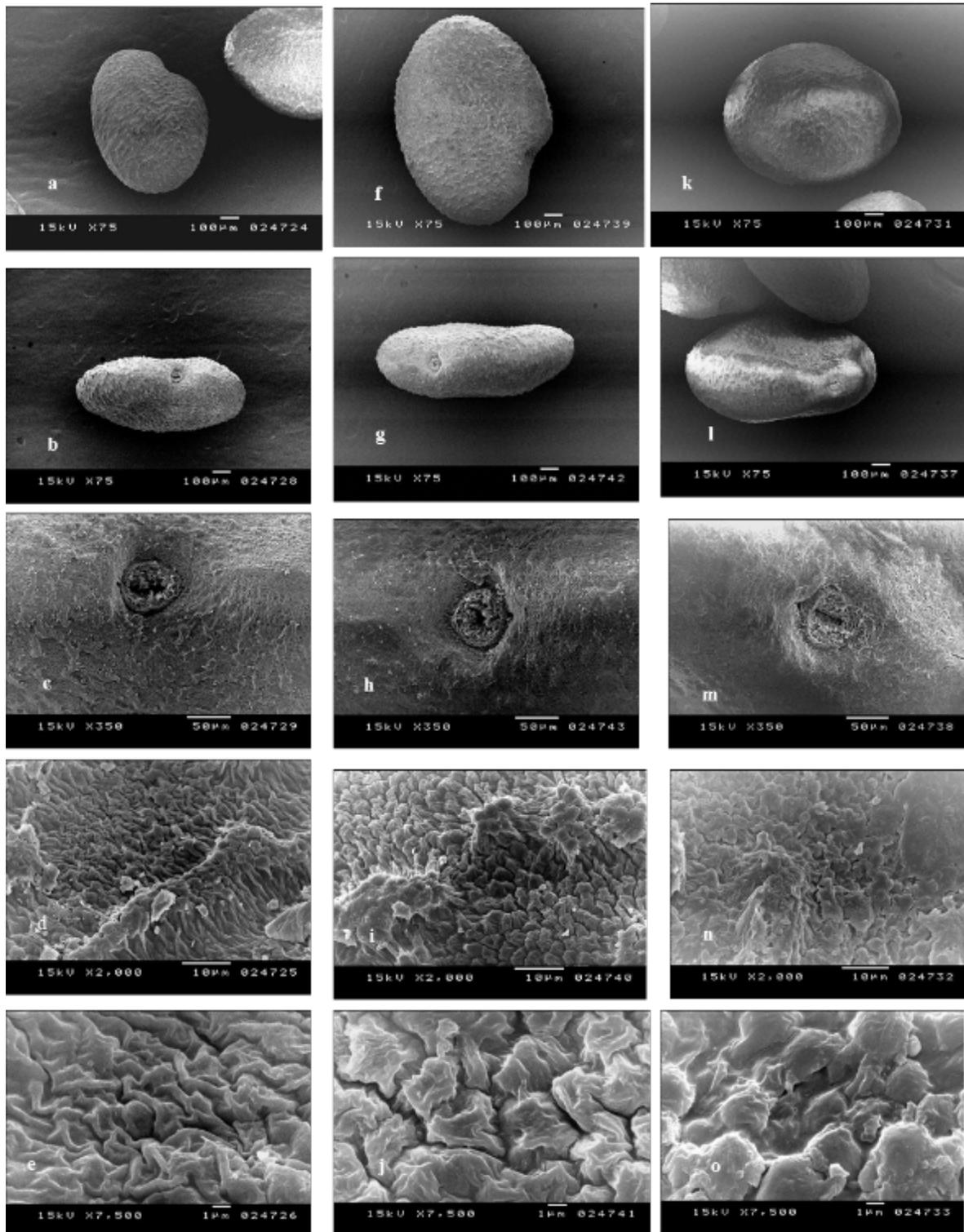


Figure III: Seed morphology and Spermoderm under SEM of *Melilotus indicus* varieties: a-e; var. *indicus*, f-j; var. *bonplandii*, k-o; var. *tommasinii* a, f, k; Seed b, g, l; Hilum position. c, h, m; Hilum shape d, l, n, e, j, o. Seed spermoderm.

2. Spermoderm anatomy (Fig. VI, Table 4).

Testa of examined taxa consists of four layers; exotesta which consists of thick elongated cells (macrosclereids) coated externally by cuticle. The second layer is light

line (*linea lucida*), after Peske & Pereira (1983), Desouza & Marsos-Filho (2001), the third layer forms hourglass cells and the fourth layer is interior parenchyma. Radicle shape cylindrical in var. *indicus*, ovate in the other varieties.

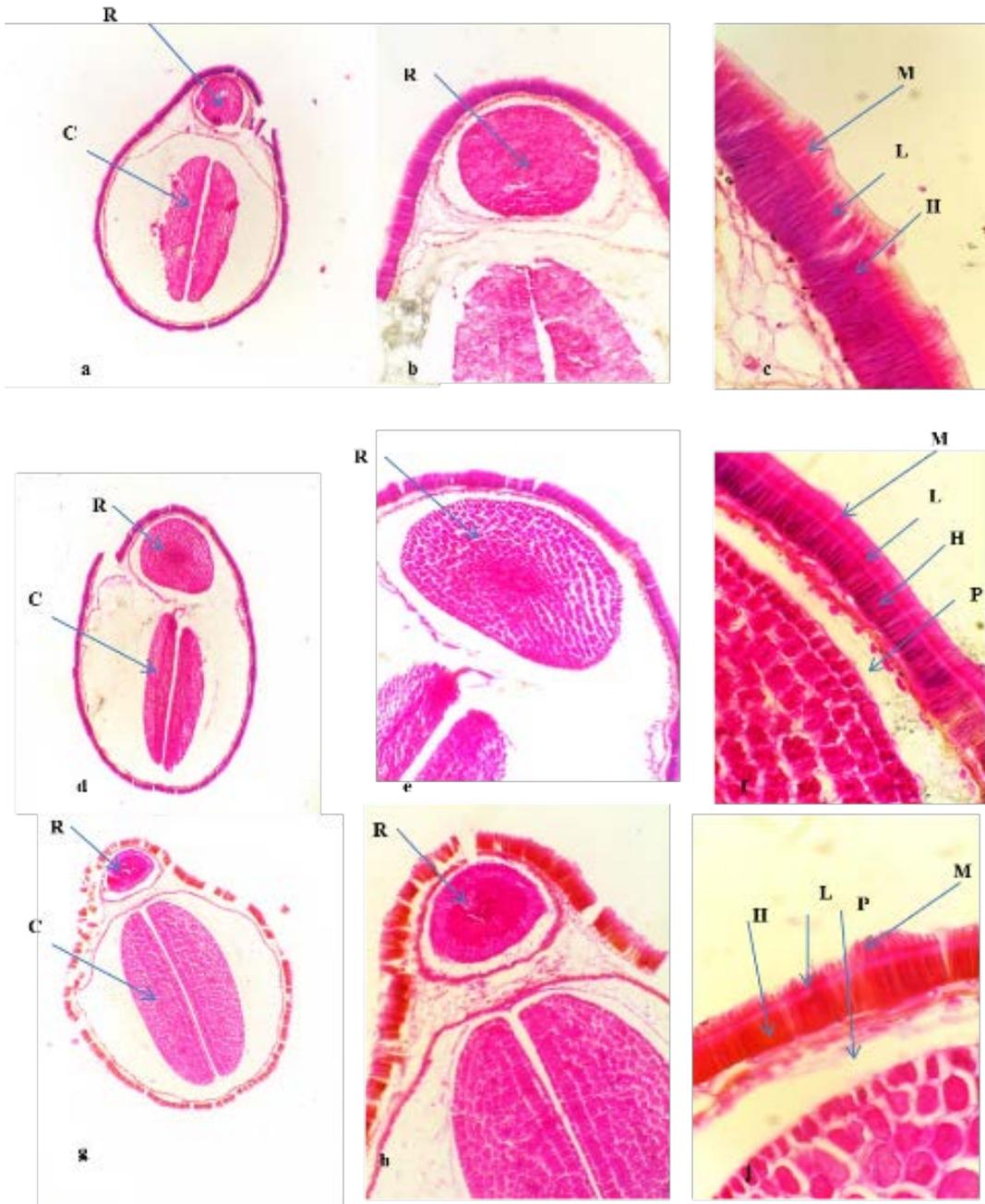


Figure IV: Speroderm anatomy as seen under LM of *Melilotus indicus* varieties: a,b,c; var *indicus*, d, e, f; var. *bonplandii*. g, h, i; var. *tommasinii*. a. L.S. in seed, b. Radicle shape, c. Testa: R, Radicle, C, Cotyledons, M. Macrosclereids, L. *Linea lucida*, H. Hourglass cells, P. Parenchyma.

Table (4): Comparison of speroderm anatomy of *M. indicus* varieties.

Characters/ Species		var. <i>indicus</i>	var. <i>bonplandii</i>	var. <i>tommasinii</i>
Cuticle Thickness μm		9	9	9
Exotesta	Thickness μm	12-15	12-15	12-15
	Macrosclereid	No. of layers	1	1
Light Layer Thickness μm		3-4	3-5	3-5
Hourglass Layer Thickness μm		37-45	30-35	35-37
Protein Layer μm		7-8	7-8	7-8
Radical	Diameter μm	350-400x450-500	450-500x300-350	270-290x380-400
	Shape	Cylindrical	Ovate	Ovate
Cotyledons	Length μm	300-375	275-290	325-340
	L c/L s* ratio	0.57	0.61	0.61
	We\ws*	0.38	0.31	0.48

*L c/L s: length of cotyledons /length of seed

*We\ws: width of cotyledons /width of seed

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In conclusion, the grouping of different varieties based on seed spermoderm, embryonic features and anatomical characters correlated well with gross morphology.

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