CHEMOTAXONOMY AND SEED MORPHOLOGICAL STUDIES OF THE GENUS MELILOTUS MILL. FROM PAKISTAN

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Abstract

The genus Melilotus Mill. of the family Fabaceae is investigated for its seed characters and chemotaxonomy. The seed morphological data, distinct proteins, peptide fingerprinting and elemental composition provide the additional tools for the specific delimitation within the genus Melilotus from Pakistan.

Key words: Melilotus, Pakistan, Seed morphology, Proteins, Elemental analysis.

Introduction

The genus Melilotus belongs to the tribe Trifolieae of the sub family Papilionoideae (Leguminosae) (Berchtold & Presl, 1820; Polhill & Raven, 1981). Since last few decades, seed morphological characters were significantly used to strengthen the taxonomic delimitation at various levels (Nasir, 1985, 1986; Quiser, 1987; Abid et al., 2015, 2016). Moreover, Capitaine (1912) studied the family Fabaceae for the seed characters and concluded that these characters were much useful for classification and identification of taxa at different levels. Similarly, Gúneş & Cirpici (2011) examined seed characteristics including surface patterns in some taxa of the genus Lathyrus from Turkey. While, Özpek et al., (2014) also studied the seed morphology of Melilotus species from Turkey. Seed characters were also used to find out the phenetic relationship between the genera of the family Fabaceae (Sahimpour et al., 2013; Khandani et al., 2016). Moreover, elemental analyses of the seeds of various species were also carried out, such as, Juknevičius & Sahienė (2007) reported the mineral elements of the various species of the family Fabaceae. While, Bahadur et al. (2011) and Ghani et al. (2014) studied the elemental analysis of fodder and medicinally important plants including the some members of the family Fabaceae. Likewise, protein and peptide fingerprinting were also found useful for the identification of various species within the family Fabaceae. (Jackson et al., 1967; Mathiesius et al., 2002). Inspite of the above mentioned reports, there is no detailed account present on seed morphology and chemotaxonomy of the genus Melilotus. Therefore, present study was under taken to provide the strength to the taxonomic delimitation of the genus Melilotus from Pakistan.

Materials and Methods

Seeds: Mature seeds of Melilotus alba, M. indica, M. messanensis and M. officinalis were collected from field as well as from herbarium specimens. Mostly 10 to 15 seeds/plants and 10 plants/species were studied (depending on the availability of material) and examined under stereomicroscope (Nikon XN Model) and scanning electron microscope (JSM-6380A). For scanning electron microscopy dry seeds were directly mounted on metallic stub using double adhesive tape and coated with gold for a period of 6 minutes in sputtering chamber and observed under SEM. The terminology used is in accordance to Berggren (1981) and Stearn (1983) with slight modifications. Seed macro and micro morphological characters viz., shape, surface patterns, colour, size and position of hilum were observed. Elemental characterization was performed by energy dispersive X-ray spectrometry (EDXS) mode which can count emission peaks to 9335 counts per second. Data was analyzed by EDS-SEM associated analytical program EDS Analysis Station.

Extraction and estimation of proteins and peptides:

Seeds (10-50) were crushed on a watch glass and transferred into 2 ml Eppendorf tubes. After grinding seeds were directly subjected for extraction in extraction buffer (i.e., 50% ACN and 1% TFA in Milli-Q water). Each extraction was performed for 1 hr at 350 rpm at 37°C using a Thermomixer comfort (Eppendorf Hamburg, Germany). The extracts were centrifuged at 14,000 rpm for 15 min (Smart 15 Micro-centrifuge Hanil, Korea) and the supernatants were collected. The supernatants were completely air dried using Speedvac concentrator-5301 (Eppendorf Hamburg, Germany) and directly reconstituted in equilibration buffer (0.1% TFA-H2O, Buffer-A). Total protein concentration of all extracts was measured by the modified dye-binding assay of Bradford (1976) using bovine serum albumin (Merck Munich, Germany) as a standard. Measurements were performed in triplicate in a microplate reader (Sunrise-Tecan Salzburg, Austria).

SDS PAGE & RP FPLC analysis:

For the proteins and peptides fingerprinting, crude seed extracts (ca. 20 µg) was subjected to SDS-PAGE analysis under dissociating and denaturing conditions (pH 8.8 in the presence of SDS and β-mercaptoethanol). The proteins and peptides were separated in 14% resolving and 5% stacking gels at 140 V for 1.5 hr and at the end of electrophoresis the gel was stained with 0.2% Colloidal Comassie Blue G-250 as recently described by Abid et al. (2014). For complementing the SDS-PAGE results, same samples (ca.
were also subjected for reversed phase fast protein liquid chromatography (AKTA-design Amersham Biosciences, Buckinghamshire, UK) using a reversed phase FPLC column (µRPC C2/C18, ST 4.6/100; Amersham Biosciences, UK). The column was equilibrated in 0.1% TFA-H2O (Buffer-A) and eluted with a short linear gradient (60% B in 20 min) of 100% ACN containing 0.1% TFA (Buffer-B). The flow rate was maintained at 1 ml/min and the elution was monitored at 280 nm. The data was analyzed by the automated software UNICORN 5.0 (Amersham Biosciences, UK).

**Observations**

**General seed characters of the genus *Melilotus***: Seeds sub-orbicular, obliquely oblong or broad elliptic, 1.9-2 x 1-1.8 mm, apex rounded, obtuse or truncate, base rounded or obtuse, brown, yellowish brown or greenish brown, surface ruminated colliculate, colliculate or verrucate, hilum lateral in position (Fig. 1; Table 1).

Represented by 4 species *M. alba* Desr., *M. indica* (L.) All., *M. messanensis* (L.) All. and *M. officinalis* (L.) Pall.

**Key to the species**

1 + Seeds yellowish brown, surface ruminated colliculate .......................................................... 2
   - Seeds greenish brown or brown, surface verrucate or colliculate ................................................ 3
2 + Seeds sub-orbicular ................................................................................................................. *M. alba*
   - Seeds broad elliptic .................................................................................................................... *M. officinalis*
3 + Seeds sub-orbicular with verrucate surface pattern ................................................................. *M. indica*
   - Seeds obliquely oblong with colliculate surface pattern ......................................................... *M. messanensis*

**Table 1. Seed morphological characters of the genus *Melilotus* Mill.**

<table>
<thead>
<tr>
<th>Name of taxa</th>
<th>Size (mm)</th>
<th>Shape</th>
<th>Apex</th>
<th>Base</th>
<th>Colour</th>
<th>Surface</th>
<th>Hilum</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Melilotus alba</em></td>
<td>2</td>
<td>Sub-Orbicular</td>
<td>Truncate</td>
<td>Rounded</td>
<td>Yellowish brown</td>
<td>Ruminated</td>
<td>Lateral</td>
</tr>
<tr>
<td><em>M. indica</em></td>
<td>1.9</td>
<td>Sub-Orbicular</td>
<td>Truncate</td>
<td>Obtuse</td>
<td>Greenish brown</td>
<td>Verrucate</td>
<td>Lateral</td>
</tr>
<tr>
<td><em>M. messanensis</em></td>
<td>1.9</td>
<td>Obliquely</td>
<td>Rounded</td>
<td>Rounded</td>
<td>Brown</td>
<td>Colliculate</td>
<td>Lateral</td>
</tr>
<tr>
<td><em>M. officinalis</em></td>
<td>2</td>
<td>Broad elliptic</td>
<td>Obtuse</td>
<td>Rounded</td>
<td>Yellowish brown</td>
<td>Ruminated</td>
<td>Lateral</td>
</tr>
</tbody>
</table>

Fig. 1. Scanning electron micrographs. *Melilotus alba*: A, seed; B, surface. *M. indica*: C, seed; D, surface. *M. messanensis*: E, seed; F, surface. *M. officinalis*: G, seed; H, surface (Scale bars: A, C, E, G=500 µm; D=50 µm; B, F, H=10 µm).
Discussion

The infra familial classification of the family Fabaceae has been the matter of interest to various scientists. To resolve the taxonomic status of the taxa within family Fabaceae several studies were carried out on different aspects such as seed morphology (Salimpour et al., 2013; Khandani et al., 2016) and chemotaxonomy like elemental analysis, protein and peptide finger printing (Jackson et al., 1967; Mathesius et al., 2002; Juknevičius & Sabienė, 2007; Ghani et al., 2014). On the basis of seed morphological data, four species of the genus Melilotus were splitted into two groups. First group, having yellowish brown seeds with ruminated colliculate surface pattern comprises M. alba and M. officinalis (Fig. 1; Table 1). While, the remaining 2 species viz., M. indica and M. messanensis are grouped by having brown or greenish brown seeds with verrucate or colliculate surface pattern. The grouping of species based on seed morphology may be well correlated with gross morphology of the genus Melilotus. Such as, M. alba and M. officinalis were reported with 3.5-7 mm long corolla and entire stipules (Ali, 1977).Whereas, M. indica and M. messanensis are characterized by having dentate stipules or entire stipules with c.2 mm long corolla (Ali, 1977). Furthermore M. indica and M. messanensis may also remain distinct having different seed shape and surface pattern, such as, M. indica is characterized with sub-orbicular seeds with verrucate pattern while, M. messanensis have obliquely oblong seeds with colliculate surface pattern (Fig. 1; Table 1). Likewise, M. alba could be separated from M. officinalis by having sub-orbicular seeds and white flowers (Ali, 1977) while M. officinalis have broad elliptic seeds and yellow flowers (Ali, 1977). The data of elemental analysis was also useful to some extent for specific delimitation as M. messanensis has P and Cu (Fig. 2; Table 2) while these elements were totally absent from rest of the species. Similarly, Na and Si were present in M. indica and M. officinalis but missing from M. alba and M. messanensis. However, the concentrations of these elements show slight variation but could not be significant enough for specific delimitation.

Seed proteins and peptides finger printing: In order to gain biochemical insight, mature seeds of M. indica, M. alba and M. officinalis were subjected to proteins and peptides finger printing analysis using 14% SDS-PAGE under dissociating-denaturing conditions (Fig. 3a). Subtle variations in proteins and peptides band pattern in molecular weight ranges from 6.5 to 66.2 kDa were observed among all the studied species (Fig. 3a). However, M. alba having more bands as compared to M. indica and M. officinalis respectively. Unfortunately, due to the small size and low protein contents in M. messanensis we could not get some results for comparison but we anticipate high intra-species variations. In order to complement the SDS-PAGE data, the same samples were also subjected to RP FPLC analysis. Very well-resolved proteins and peptides separation profiles were observed (Fig. 3b) which confirmed our aforementioned results. The three species M. indica, M. alba and M. officinalis could be ideally distinguished from each other at the end of elution profiles by high molecular mass protein fractions (retention time 15-20 min). Likewise, the subtle differences among the three species have also been observed in the peak pattern of hydrophilic peptides in the unbound region (i.e. retention time 0–7 min).

Table 2. Elemental composition of the seeds of Melilotus species determined using energy-dispersive X-ray spectroscopy (EDXS) using the associated analytical program EDS analysis station.

<table>
<thead>
<tr>
<th>Elements</th>
<th>M. indica (% mass)</th>
<th>M. alba (% mass)**</th>
<th>M. officinalis</th>
<th>M. messanensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>47.12</td>
<td>49.71</td>
<td>50.23</td>
<td>48.76</td>
</tr>
<tr>
<td>O</td>
<td>42.07</td>
<td>45.10</td>
<td>43.20</td>
<td>36.65</td>
</tr>
<tr>
<td>Na</td>
<td>2.84</td>
<td>-</td>
<td>0.63</td>
<td>-</td>
</tr>
<tr>
<td>Mg</td>
<td>0.45</td>
<td>0.41</td>
<td>0.56</td>
<td>1.41</td>
</tr>
<tr>
<td>Al</td>
<td>0.54</td>
<td>0.86</td>
<td>0.78</td>
<td>0.4</td>
</tr>
<tr>
<td>Si</td>
<td>0.64</td>
<td>-</td>
<td>1.86</td>
<td>-</td>
</tr>
<tr>
<td>Ca</td>
<td>1.57</td>
<td>0.92</td>
<td>1.86</td>
<td>2.67</td>
</tr>
<tr>
<td>K</td>
<td>4.22</td>
<td>3.69</td>
<td>2.67</td>
<td>8.25</td>
</tr>
<tr>
<td>Cl</td>
<td>2.44</td>
<td>0.42</td>
<td>0.38</td>
<td>1.14</td>
</tr>
<tr>
<td>P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.71</td>
</tr>
<tr>
<td>Cu</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.49</td>
</tr>
</tbody>
</table>

**Results presented are means of three independent measurements
Fig. 3a. Proteins and peptides figure printing of the seeds of *Melilotus* species using 14% polyacrylamide gel electrophoresis under dissociating and denaturing (pH 8.8 in presence of SDS and β-mercaptoethanol) conditions.

Fig. 3b. RP FPLC analysis of the same samples on µRPC C2/C18 column. Asterisks indicates the subtle variations in band pattern and their elution profiles, respectively.

References


Nasir, Y.J. 1985. Seed Studied in the *Androsace* L. (Primulaceae) species Found


(Received for publication 15 January 2017)