Differentiation of Different Species of *Origanum* and *Thymus* using Proteins and Isoenzymes Profile

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**Abstract:** Different species/populations of *Origanum* and *Thymus* indigenous to Lebanon were analyzed by slab gel electrophoresis to compare protein patterns and isoenzymes phenotypes of esterase and peroxidase. Multiple electromorphs were obtained. The differences in the esterase profiles obtained by electrophoresis were consistent with the results of morphological identification of different groups of *Origanum* from different geographical areas. The esterase patterns successfully differentiate between different phenotypes/species of *Origanum* also between two different species of *Thymus*. The results demonstrated that isoenzymes phenotypes are useful to supplement the morphological characterisation of these species. The highest esterase activities and clearer banding profile were obtained during and after flowering period of plant development.

**Key words:** Electrophoresis, enzymes, esterase, *Origanum*, protein, taxonomy, *Thymus*

**INTRODUCTION**

The usefulness of isoenzyme species identification, assessing genetic distance between taxa and analysing systematic relationships have been demonstrated in different areas: bacteria (Hoskins et al., 1992; Medina et al., 2004), fungi (Bach and Kimati, 2004), nematodes (Ibrahim et al., 1995, 1997), insects (Stasinakis et al., 2001) and in a large number of plants (Cooke, 1984; Ibrahim, 1991; Ibrahim and Perry, 1993; Collet et al., 2005; Ganjewa and Luthra, 2007). The genus *Origanum* is a member of the Lamiaceae family which is widely distributed in Mediterranean area and Northern Africa (Jetswaart, 1980; Kokkini et al., 1997). Most of commercial oregano comes from wild populations without focusing on specific subspecies (Oliveir, 1997). This genus includes numerous species, subspecies, varieties and hybrids that cannot be distinguished very easily. De Martino et al. (2009) reported the difficulties to differentiate between different subspecies of *O. vulgare* using morphological aspects alone. Kokkini et al. (1997) also stated that *O. vulgare* has very variable taxon both in morphological and in chemical features. Based on the presence of essential oils there are intraspecific taxa of oregano that exhibit no "oregano" character (Bernard, 1997).

*Origanum* plays an important role among temperate culinary herbs in world trade (D’Antuono et al., 2000). *Origanum* is the main constituent of perfumes and other cosmetic products and is used to improve storage stability in food sectors. Also, it has been found to possess significant functions such as antioxidant, antifungal, antibacterial, insecticidal and nematicidal properties (Oka et al., 2001; Burt, 2004; Kulisevic et al., 2004; Ibrahim et al., 2006, 2011; Bakkali et al., 2008). Despite its economic importance, its genetic resources and variability potential for utilization have not yet been fully explored. Lebanese flora is known to be rich with medicinal and aromatic plants (Nehmeh, 1978). However, there is no or little information exists regarding taxonomic identification, morphological, phenological, genetic and chemical characteristics of the grown and wild species of *Origanum* in the country. The objectives of this study were: (1) to differentiate between different population/species of *Origanum* and thyme collected from different areas using protein and isoenzymes profile, (2) to evaluate esterase activity of *Origanum* species before, during and after flowering.

**MATERIALS AND METHODS**

**Plant material:** Over 53 different samples of *Origanum* and *Thymus* plants (including soil) were collected from 36 different regions, areas, sites and altitudes of Lebanon (Table 1) during March-May 2009. All samples were divided into two parts. One part was potted using the same soil brought from the site of sampling and cultured at the Department of Plant Protection. Second part was cultured in a field at Gazeer Research Station, Faculty of Agricultural and Veterinary Sciences. Samples of *Origanum* populations were grouped according to their morphological characteristics (Farias et al., 2010).
Table 1: Showing the region, place and altitude of collected samples of *Origanum* and *Thymus* in Lebanon

<table>
<thead>
<tr>
<th>Region</th>
<th>Place</th>
<th>Altitude (m)</th>
<th>Code</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>South Lebanon</td>
<td>Jezeine</td>
<td>950</td>
<td>27</td>
<td>VI</td>
</tr>
<tr>
<td></td>
<td>Chehour</td>
<td>320</td>
<td>25</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Harbaya</td>
<td>750</td>
<td>5</td>
<td>VIII</td>
</tr>
<tr>
<td></td>
<td>El khiam</td>
<td>695</td>
<td>26</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>El douir</td>
<td>370</td>
<td>28</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>Kfarhamam</td>
<td>800</td>
<td>2</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>Bassouryeh</td>
<td>170</td>
<td>4</td>
<td>VII</td>
</tr>
<tr>
<td>North Lebanon</td>
<td>Shikha</td>
<td>20</td>
<td>11</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Hadhour</td>
<td>1030</td>
<td>15</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Bcherri</td>
<td>1460</td>
<td>13</td>
<td>VIII</td>
</tr>
<tr>
<td></td>
<td>Ouamatt akkar</td>
<td>350</td>
<td>17</td>
<td>III</td>
</tr>
<tr>
<td>Mount Lebanon</td>
<td>Birk l kesruan</td>
<td>240</td>
<td>10</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Chosai Al-matan</td>
<td>530</td>
<td>23</td>
<td>VIII</td>
</tr>
<tr>
<td></td>
<td>Barouk</td>
<td>1110</td>
<td>34</td>
<td>VIII</td>
</tr>
<tr>
<td></td>
<td>Bsaba (Babda)</td>
<td>330</td>
<td>24</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Bsaba (Babda)</td>
<td>330</td>
<td>18</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Naher Ibrahim</td>
<td>58</td>
<td>12</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Medref</td>
<td>280</td>
<td>1</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>Kharbheen kesruan</td>
<td>1250</td>
<td>7</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Moultrine jehil</td>
<td>15</td>
<td>9</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Kfour kesruan</td>
<td>830</td>
<td>21</td>
<td>VIII</td>
</tr>
<tr>
<td>Bekaa</td>
<td>Kherbet kamarf (plain)</td>
<td>1080</td>
<td>35</td>
<td>VIII</td>
</tr>
<tr>
<td></td>
<td>Nabi chtit</td>
<td>1289</td>
<td>33</td>
<td>XIX</td>
</tr>
<tr>
<td></td>
<td>Kherbet kamarf (moorage)</td>
<td>1100</td>
<td>36</td>
<td>VIII</td>
</tr>
</tbody>
</table>

*-Thymus capitatus* (IX) and *T. herbae (X)*

Protein and enzymes staining: For general protein patterns, gels were stained with 0.5% coomassie brilliant blue R250 in 25% ethanol and 10% acetic acid at 45°C for one hour. Coomassie-stained gels were subsequently stained with several changes of 50% ethanol and 7% acetic acid.

For non-specific α and β esterases, gels were incubated at 37°C in the dark for 30-40 min in a solution of 100 mg Fast Blue RR Salt, 50 µg α-, β-naphthyl acetate and 50 µg α-naphthyl butyrate dissolved in 5 ml acetonitrile up to 100 mL with 0.2 M Tris-HCl buffer, pH 6.6. The substrates were filtered through filter paper and used immediately. The reaction was stopped by adding 10% acetic acid. Relative electrophoresis mobility (Rm) for each enzyme band was calculated as the ratio of its movement to that of the marker dye.

Peroxidase activity was visualized by incubating the gels for 5 min at room temperature in a mixture of 0.5% (w/v) benzidine dissolved in 10 mL of acetic acid and made up to 100 mL with distilled water. They were then placed in distilled water containing 0.3% (v/v) hydrogen peroxide (H₂O₂).

Esterase activity using spectrophotometry: Esterase activity was detected using the same solution as for gel electrophoresis. Reading was done at 600 nm on a Beckman DU-70 spectrophotometer. Samples were incubated in the dark at 37°C and reading was taken at 0, 5, 10 and 20 min intervals.

RESULTS

The results of native polyacrylamide gel technique (protein banding profiles) are presented in Fig. 1 and 2. A total of 13 bands were detected among the population of *Origanum* (I-VIII) and in thyme (*IX*) (Fig. 1). Some bands were common for both *Origanum* and *Thymus*. For example, band at Rm = 0.11 shared between group V, I, IX. Not all extracts from collected plants before flowering showed clear banding pattern. However, when the gel was run using plant extracts from flowering plant, a more clear banding pattern was obtained (Fig. 2). A total of 41 bands were detected. There was clear difference between the banding patterns of each group of *Origanum* by several distinct bands. A common band at Rm = 0.18 was detected in all the groups tested. Band at Rm = 0.24 was present in group II, I, V, VI, III. Another distinct band (Rm = 0.33) was present in all population of *Origanum* except in V, VII group. None of the *Origanum* groups shared the same protein profile indicating clear chemical differences. The quantification of proteins in *Origanum* showed numerous bands suggesting that protein profiles are less easy to
use for diagnostic purposes than isoenzymes phenotypes. The total protein assay using Bradford’s method revealed the difference in the concentration of protein between different populations of *Origanum* with an average of 0.015 g/g of dry material. The Kjeldahl methods also demonstrated the differences in the percentage of protein content among all the populations tested ranged between 10.9-11.9%. 

Analysis of non-specific esterase from different populations of *Origanum* gave distinct esterase phenotypes for all the tested groups of *Origanum* and the two different species of thyme (Fig. 3). The gel failed to give clear banding profile before flowering (results not shown). However, much better banding pattern was detected during the flowering development of the plants (Fig. 3). Esterase analysis revealed six distinct phenotypes and the bands could be divided into three electromorphic groups. The first group of slowly migrating Est A bands (Rm = 0.07 to Rm = 0.18) were particularly evident in group II, III, VII, X1, Ft, whereas these bands were absent in V, VIII, I, and Ft groups. The second group of moderately migrating bands Est B (Rm = 0.18 to Rm = 0.25) have moderately stained in groups V, II, VIII, I, and Ft but were absent in all the remaining groups. The third group Est C of rapidly migrating bands (Rm = 0.28 to Rm = 0.3) were found in V, VIII, I, X1, Ft, X2 and Ft groups. The esterase activity for all the groups indicated the existence of three active alleles.

The results of peroxidase analysis also revealed distinct phenotypic patterns for most of the tested groups (Fig. 4) ranging from one to three bands. However, some of these bands faded very quickly. Only one single strong band stained in group II VIII, I, X1 but quickly disappeared in other groups. The peroxidase analysis also distinguished between different groups of *Origanum* and *Thymus* species indicating clear different phenotypic patterns.

The results of esterase activity using spectrophotometry are presented in Fig. 5. The esterase activity ranged between 0.14 to 0.62 nm in *Origanum* species and slightly lower at 0.12 to 0.56 nm in *Thymus*. The highest esterase activity was detected sooner after flowering period of plant development. The two species showed almost the same activity but slightly higher in *Origanum* sp. (Fig. 5).

**DISCUSSION**

Protein and isoenzymes banding patterns revealed distinct differences among different groups/species of *Origanum* and between *Origanum* and *Thymus* collected from different region of Lebanon. The protein profile differed between different groups but there were some
species/population-specific bands. Although, the protein banding pattern discriminated between different groups of *Origanum* and other species of thyme, the multienzyme phenotypes proved to be very useful for differentiating between interspecies and species. Although the current study is the first to report the use of esterase enzymes for the identification of different population/species of *Origanum* and *Thymus*, several studies have also demonstrated the usefulness of isoenzyme phenotypes to support and extend taxonomic characterisation (Hoskins et al., 1992; Medina et al., 2004; Bach and Kimati, 2004; Ibrahim, 1991; Ibrahim and Perry, 1993; Ibrahim et al., 1995, 1997; Stasiakis et al., 2001; Collet et al., 2005; Ganjewa and Luthra, 2007). Several studies used essential oil to differentiate between different populations of *Origanum vulgare* (De Martino et al., 2009). The post-electrophoretic detection of esterases is a sensitive technique applied in bacterial systems that mainly provides information on the similarity of strains within the same species or subspecies according to their esterase pattern (Medina et al., 2004). Generally, chemotypes form biochemical varieties or “physiological forms in botanical species, each of which has a specific enzymatic equipment (De Martino et al., 2009). In this study the electrophoresis results revealed that the highest esterase activities were present during the flowering period of growth, this was also supported by spectrophotometry analysis were the highest activities during or just soon after flowering period of plant development. Esterase activity was used as a growth marker in tobacco and Norway spruce (Vitek et al., 2004). The phenols content, generally, is high during flowering stage in phenol-type *Origanum* plant (Werker et al., 1985; Putievsky et al., 1988). The highest concentration of thymol and carvacrol was detected after flowering in both wild and cultivated

*Origanum syriacum* (Zein et al., 2011) the proportion of carvacrol has been shown to be much higher in the summer, whereas *p*-cymene predominates in the autumn (Kokkini et al., 1997; Senatore, 1996; Jerkovic et al., 2001). In present study the esterase activities were also high just after flowering. The quality and quantity of essential oil composition can vary according to climate, soil composition, geographical location, seasonal variation, plant organ, age and vegetative cycle stage and harvesting time (Abu-Lafi et al., 2007, 2008). Vural (2009) reported that when fresh or frozen leaves of plants collected in autumn were used for the isolation of DNA, no positive result in PCR reaction was obtained regardless of the isolation protocol being used. In our results the protein and isoenzymes pattern were not successful when the plant material was collected before flowering (winter time). This was probably due to the accumulation of large amounts of secondary metabolites in old plant material, as previously reported (Khanuja et al., 1999) or the high amounts of oils. The use of this technique for biochemical taxonomy of different species of *Origanum* and *Thymus* may provide useful species-specific enzyme markers. It is now important to determine the variation in enzyme bands between geographically isolated populations of the same species.

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REFERENCES


