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

S.K. Ibrahim, L. Ibrahim, A. Ismail, A. Basal, M. Kayal, H. Ghanem and S. Rammel
Faculty of Agricultural Sciences, Lebanese University, Beirut, Lebanon

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 (Ietswaart, 1980; Kokkini *et al*., 1997). Most of commercial oregano comes from wild populations without focusing on specific subspecies (Olivier, 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 (Bernath, 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; Kulisic *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). One

Corresponding Author: Said K. Ibrahim, Faculty of Agricultural Sciences, Lebanese University, Beirut, Lebanon

Table 1: Showing the region, place and altitude of collected samples of *Origanum* and *Thymus* in Lebanon

Region	Place	Altitude/m	Code	Group
South Lebanon	Jezzine	950	27	VI
	Chehour	320	25	I
	Hasbayya	750	5[c]	VIII
	El khiam	695	26	V
	El doueir	370	28	V
	Kfarhamam	800	2[c]	V
	Bazouriyeh	170	4	VII
North Lebanon	Shikka	20	11[b]	III
	Hadtoun	1030	15	II
	Bcherri	1400	13	VIII
	Ouainat akkar	350	17	III
Mount Lebanon	Bkirki keserouan	240	10[b]	III
	Chaouie Al-maten	530	23	VIII
	Barouk	1110	34	VIII
	Bsaba (Baabda)	330	24*	Xt
	Bsaba (Baabda)	330	18	III
	Naher ibrahim	38	12[b]	IV
	Mechref	280	1[c]	VIII
	Kfarthebian keserouan	1250	7[e]	III
	Mouhrine jbeil	15	9[d]	II
	Kfour keserouan	830	21[a]	VIII
Bekaa	Kherbet kanafar (plain)	1090	35	VIII
	Nabi chit	1289	33*	IXt
	Kherbet kanafar (montagne)	1100	36	VIII

*-*Thymus capitatus* (IXt) and *T. hirsutus* (Xt)

representative from each group was used for biochemical analysis (Table 1).

Extraction of plant proteins: 100 mg of fresh leaves/flowers were homogenised in pestle and mortar with cold 0.1 M Tris.-HCI buffer (pH 7.8) containing 20% glycerol, 2% Tween-20 and mercoptoethenol. The extracts were filtrated using muslin and the supernatant transferred into Eppendorff tube for centrifugation at 13000 rpm for 10 min at 4°C. The 25-30 μL clarified supernatant was introduced immediately into the electrophoresis cell. The concentration of proteins in the extracts was estimated by Bradford (1976) method, using bovine serum albumin (BSA, Sigma) as the standard.

Polyacrylamide gel electrophoresis: The procedures used for gel electrophoresis of protein and enzymes have been described previously (Ibrahim and Perry, 1993). Briefly, the isoenzymes present in plant samples were separated by native polyacrylamide gel electrophoresis in mini-slab gel apparatus using gel size 90 mm wide, 80 mm long X 1 mm thick. 3% acrylamide stacking gel and 7% acrylamide separation gel were used. Samples of 25-30 μL were injected into wells formed in the stacking gel. The buffer system was essentially that of Laemmli (1970), except that Tween-20 was substituted for Sodium Dodecyl Sulphate (SDS). A constant current 20 mA was applied throughout the run.

Protein and enzymes staining: For general protein patterns, gels were stained with 0.5% coomassie brillliant blue R250 in 25% ethanol and 10% acetic acid at 45°C for one hour. Coomassie-stained gels were subsequently distained 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 acetone made 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 (H_2O_2).

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 (IXt) (Fig. 1). Some bands were common for both *Origanum* and *Thymus*. For example, band at Rm = 0.11 shared between group V, I, IXt. 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, 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

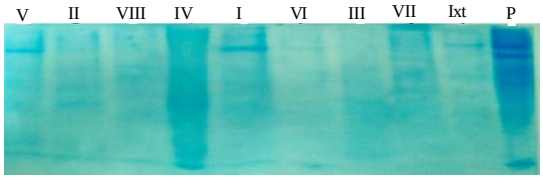

Fig. 1: Protein profile of different population of *Origanum* (I-VIII) and *Thymus capitatus* (IXt) before flowering, p-*Pisum sativum* (Control)

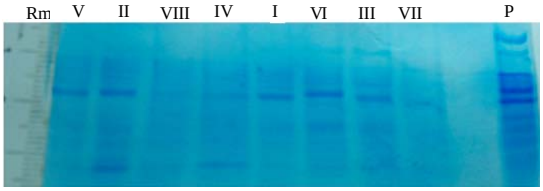

Fig. 2: Protein profile of different populations of *Origanum* (I-VIII group) during flowering, p-*Pisum sativum* plant (Control). Rm- relative mobility

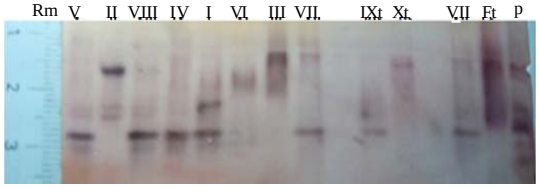

Fig. 3: Esterase isoenzymes profile of different population of *Origanum* (1-VII) and *Thymus capitatus* (IXt) and *T. hirsutus* (Xt), (Frensh Oregano, Unidentified) during flowering, p-*Pisum sativum* (Control). Rm- relative mobility

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, Xt, Ft), whereas these bands were absent in V,VIII,IV,VII and IXt 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, IV, I, VII, IXt, Xt and Ft groups. The esterase activity for all the groups indicated the existence of three active alleles.

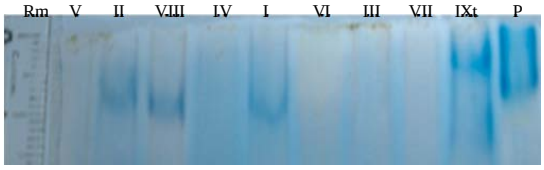

Fig. 4: Peroxidase isoenzymes profile of different population of *Origanum* (I-VIII) and *Thymus capitatus* (IXt) during flowering, p-*Pisum sativum* (Control). Rm- relative mobility

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, IXt 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

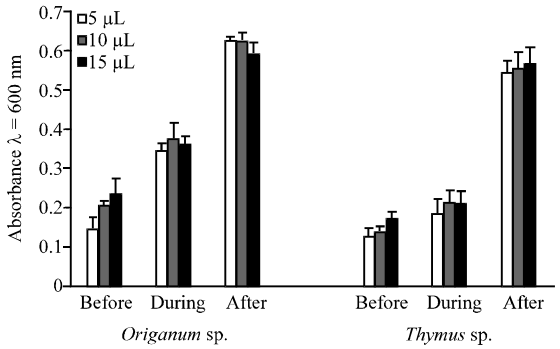

Fig. 5: Esterase activity before, during and after flowering of *Origanum* and *Thymus* after 10 min incubation. Bars = ±SE

species/population-specific bands. Although, the protein banding pattern discriminated between different groups of *Origanum* and other species of thyme, the multienzymes 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; Stasinakis *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 (Vitecek *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; Jerkovixc *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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