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Studies on intra-specific variation in a multipotent medicinal plant *Ocimum sanctum* Linn. using isozymes

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ABSTRACT

Objective: To analyze the similarity and variation among different populations of *Ocimum sanctum* (*O. sanctum*) by isoperoxidase, isoesterase, acid phosphatase and alkaline phosphatase profile. **Methods:** The isolation, separations and staining of the enzymes isoperoxidase, isoesterase, acid phosphatase and alkaline phosphatase were performed as described by Sadasivam and Manickam. **Results:** In isoperoxidase system, the band PRX4[1] (0.36) was present only in the accession collected from Salem, similar to that band PRX4[2] showed its presence only in the accession collected from Kolli Hills and PRX5[1] (0.42) was expressed only in Wyanad accession. The accession collected from Tirunelveli showed its unique banding profile in region ACP2[3] with 0.18 molecular weight-Rf values in the acid phospahatase system. In esterase system, EST5[1] and 8[1] were observed only in the accession collected from Tenkasi. In the alkaline phosphatase enzyme system, only one region (AKP6) of activity with single band (AKP6[1]-0.51) was observed. In this enzyme system, all the selected accession showed their expression. Highest percentage (0.666 7) of similarity was observed between the accessions collected from Tirunelveli and Kolli Hills, Nagercoil and Kolli Hills and Salem and Andaman while the highest percentage (0.800 0) of variation was observed between the accessions collected from Salem and Andaman. The isozyme profile based cladogram showed two clusters, cluster 1 (C1) included the accessions collected from Tenkasi, Tirunelveli, Nagercoil and Kolli Hills and they showed highest percentage of divergence with the other three accessions. The cluster 2 (C2) included the accessions collected from Wyanad, Salem and Andaman. **Conclusions:** Unique banding profiles of esterase, peroxidase, acid phosphatase and alkaline phospahatase were observed in *O. sanctum*, which represent the fingerprint of medicinally important plant. Such finger printing is useful in differentiating the species and act as biochemical markers for these species in plant systematic studies in the near future.

1. Introduction

Medicinal plants are a source of great economic value all over the world. Nature has bestowed on us a very rich botanical wealth and a large number of diverse types of plants grow in different parts of the country. India is rich in all the three levels of biodiversity *viz*., species diversity, genetic diversity and habitat diversity. In India thousands of species are known to have medicinal value and the use of different parts of several medicinal plants to cure specific ailments has been in vogue since ancient times. *Ocimum* respectively named Holy basil is an aromatic herb that has been used traditionally as medicinal herb in the treatment of headaches, cough, diarrhea, constipation, warts, worms and kidney malfunctions[1]. *Ocimum sanctum* (*O. sanctum*) the sacred 'Tulsi' finds diverse uses in the indigenous system of medicine. The leaves of the plant have been used as an expectorant, diaphoretic, anticancer, antihelminthic, antiseptic, analgesic and tonic rejuvenator[2,3]. Dry leaves are used in fungal infections, the fresh juice of the leaves are used in the treatment of bronchitis, otitis media, and skin diseases[4]. Essential oil of *O. sanctum* has also showed antifungal activity against *Aspergillus niger*, *Rhizopus stolinifera*, and *Penicillium digitatum*[5]. Alcoholic and aqueous extract of *O. sanctum* have also shown adaptogenic action against antigenic challenge of *Salmonella typhi* and sheep erythrocytes[6]. *O. sanctum* also have therapeutic effects for nasal polyps[7], an upper respiratory tract diseases

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and it has been used as a bathing solution for treatment of ulcers. It is also a source of aroma compounds and essential oils containing biological active constituents that posses insecticidal, nematicidal, fungistatic and antimicrobial properties[8-13]. *Ocimum* species contain a wide range of essential oils rich in phenolic compounds and a wide array of other natural products including polyphenols such as flavonoids are potent antioxidants, free radicals scavengers and metal chelator[14]. It is an important medicinal plant in India and Bangladesh and used as herb against a number of diseases leucoderma, strangury, lumbago, kapha and vata asthma, bronchitis, vomiting[15]. The bruised fresh roots stem and leaves are applied to the bites of mosquitoes[2]. Because of the important of this species, numerous studies have been carried out on phytochemical and pharmacological, antimicrobial, micorpropgation and genetic diversity using random amplification of polymorphic DNA[16-20]. Inter-specific genetic relationships among seven different species of *Ocimum* has been analysed by Harisaranraj *et al*[21]. But there is no report on inter-specific genetic diversity in *O. sanctum* from South India, from where large quantity of leaves of this plant is exported. Isozyme analysis is a highly appropriate method for identifying genomic allele components as well as supplementing DNA analysis. Since the 1930s, electrophoresis in conjunction with the zymogram technique has been used as a tool for the study of heritable variation. Isozymes are widely used because of their relative efficiency and cost effectiveness, particularly in studies of intra- and inter-specific variation[22,23]. Isozymes are practical and useful genetic and biochemical markers as well as good estimators of genetic variability in plant populations[24,25]. The influence of peroxidase in the regulation of cell growth and differentiation has been observed by Johnson[26]. Recently, studies on protein and isoenzyme homologies have been carried out along with the improved biochemical techniques. Leaves of other species of *Ocimum* are often adulterated with this species. The analysis of isozyme pattern has become a useful tool for the investigation of variations in plant populations[27-29] and thus it is useful to differentiate the adulterants by using molecular marker. With this background the present study was aimed to elucidate the genetic variations among the populations (Tenkasi, Tirunelveli, Nagercoil, Salem, Kolli Hills, Wyanad and Andaman) of *O. sanctum* using isozymic profiles.

2. Materials and methods

Live plants from different populations of *O. sanctum* were collected from seven localities (accessions) and were established in the green house attached to Department of Plant Biology and Plant Biotechnology, St. Xavier's College (Autonomous), Palayamkottai, India. For enzyme extraction, 500 to 1 000 mg of freshly harvested young leaves were taken and homogenized with 3.5 mL of ice-cold homogenizing buffer in a pre-chilled pestle and mortar. For peroxidase, the young shoots were homogenized with 0.1M phosphate buffer (pH 7.0) and centrifuged at 12 000 rpm for 10 min. For esterase, the young leaves were collected and ground with pre-chilled isolation buffer (0.1M phosphate buffer pH 9.2) and centrifuged at 12 000 rpm for 10 min. For acid and alkaline phosphatase the young leaves were harvested and homogenized in a mortar and pestle with citrate buffer and centrifuged at 20 000 rpm for 10 min. The supernatant was subjected to electrophoresis as described by Sadasivam and Manickam[30] on poly acrylamide gel electrophoresis as per Sadasivam and Manickam[30]. Staining solutions for isoperoxidase, isoesterasse, acid phosphatase and alkaline phosphatase were prepared as per Sadasivam and Manickam[30] for the detection of iso-enzymes. After the electrophoresis, the gels were incubated in the staining solution for few minutes under dark condition till the clear bands appeared. The gels were fixed with 7% acetic acid solution for 30 min, washed with distilled water and photographed using the gel documentation system manufactured by Biotech, Yercaud, India. Pairing affinity or similarity index was calculated by the method described by Sokal and Sneath[31]. Similarity indices of isoenzyme system were generated from the banding pattern thus obtained. Based on the isoenzyme banding profile the zymogram was constructed.

3. Results

3.1. Peroxidase (PRX)

A total of sixteen bands in seven various position and six regions of activity (PRX 1, 3, 4, 5, 7 and 8) were observed in this enzyme system. The accessions collected from Salem, Kolli Hills and Wyanad showed their unique presence in the isoperoxidase system. The band $PRX4^{1}$ (0.36) was present only in the accession collected from Salem, similar to that band $PRX4^{2}$ was showed its presence only in the accession collected from Kolli Hills and $PRX5^{1}$ (0.42) was expressed only in Wyanad accession. Band ($PRX1^{1}$) with Rf value 0.10 was shared by the accessions collected from Tenkasi, Tirunelveli, Kolli Hills and Wyanad, similar to that the band $PRX7^{1}$ was also expressed in the accessions collected from Tenkasi, Tinelveli, Wyanad and Andaman. Next to that, O. sancum collected from Nagercoil, Wyanad and Andaman accessions showed their similarity by the presence of band $PRX3^{1}$ with the Rf value 0.30 in these isoperoxidase system. The accession collected from Nagercoil and Salem showed their jointly presence in the banding profile, $PRX8^{1}$ with the Rf value 0.76 in the isoperoxidase system (Table 1, Figure 1 A).

3.2. Acid phosphatase (ACP)

Multiple regions (6) of activity were obtained for this enzyme system ACP 1 to 5 and 9. The accession collected from Tirunelveli showed its unique banding profile in region $ACP2^{3}$ with 0.18 molecular weight (MW)-Rf values. The band $ACP9^{1}$ (0.81) showed its universal presence in all the accessions. $ACP1^{1}$ (0.08) was shared by the accessions collected from Salem, Wyanad and Andaman. Similar to

Table 1.
MW-Rf values and banding profile of *O. sanctum*.

Positions		MW-Rf	TEN	TVL	NGL	KH	SAL	WY	AN
Iso-peroxidase	PRX1[1]	0.10	+	+	−	+	−	+	−
	PRX3[1]	0.30	−	−	+	−	−	+	+
	PRX4[1]	0.36	−	−	−	−	+	−	−
	PRX4[2]	0.40	−	−	−	+	−	−	−
	PRX5[1]	0.42	−	−	−	−	−	+	−
	PRX7[1]	0.70	+	+	−	−	−	+	+
	PRX8[1]	0.76	−	−	+	−	+	−	−
Acid phosphatase	ACP1[1]	0.08	−	−	−	−	+	+	+
	ACP2[1]	0.11	−	−	−	−	−	+	+
	ACP2[2]	0.15	+	−	−	−	+	−	−
	ACP2[3]	0.18	−	+	−	−	−	−	−
	ACP2[4]	0.20	−	−	−	−	−	+	+
	ACP3[1]	0.26	+	−	−	−	+	−	−
	ACP4[1]	0.34	+	+	+	−	−	−	−
	ACP5[1]	0.42	−	−	+	+	−	−	−
	ACP9[1]	0.81	+	+	+	+	+	+	+
Alkaline phosphatase Esterase	AKP6[1]	0.51	+	+	+	+	+	+	+
	EST1[1]	0.08	+	−	−	−	+	+	+
	EST3[1]	0.28	−	−	−	−	+	+	+
	EST4[1]	0.31	−	−	−	−	+	+	+
	EST4[2]	0.34	−	+	+	+	−	−	−
	EST5[1]	0.46	+	−	−	−	−	−	−
	EST6[1]	0.51	−	−	−	−	+	+	+
	EST7[1]	0.61	−	−	−	−	+	+	+
	EST8[1]	0.78	+	−	−	−	−	−	−
	EST9[1]	0.81	−	+	+	+	−	−	−

TEN-Tenkasi; TVL-Tirunelveli; NGL-Nagercoil, KH-Kolli Hills; SAL-Salem; WY-Wyanad; AN-Andaman.

Table 2.
Combined similarity indices of isoenzyme systems of *O. sanctum*.

Accessions	TEN	TVL	NGL	KH	SAL	WY	AN
TEN	1.000 0						
TVL	0.555 6	1.000 0					
NGL	0.333 3	0.625 0	1.000 0				
KH	0.352 9	0.666 7	0.666 7	1.000 0			
SAL	0.454 5	0.200 0	0.300 0	0.210 5	1.000 0		
WY	0.416 7	0.363 6	0.272 7	0.285 7	0.615 4	1.000 0	
AN	0.272 7	0.300 0	0.300 0	0.210 5	0.666 7	0.315 8	1.0000 0

TEN-Tenkasi; TVL-Tirunelveli; NGL-Nagercoil, KH-Kolli Hills; SAL-Salem; WY-Wyanad; AN-Andaman.

that the accessions collected from Tenkasi, Tirunelveli and Nagercoil also shared the band ACP4[1] with 0.34 MW-Rf values. Next to that, the bands ACP2[1] (0.11) and ACP2[4] (0.20) were shared by the accessions collected from Wyanad and Andaman. The bands ACP2[2] (0.15) and ACP3[1] (0.26) were showed their jointly presence in the accessions collected from Tenkasi and Salem. Similar to that the accessions collected from Nagercoil and Kolli Hills shared a band ACP5[1] (0.42) in this enzyme system (Table 1, Figure 1 D).

3.3. Esterase (EST)

In the esterase enzyme system, nine regions (EST1, 3-9) of activity were obtained. A total of twenty four bands and nine different positions were observed in the enzyme system of *O. sanctum*. EST3[1](0.28), 4[1] (0.31), 6[1] (0.51) and 7[1] (0.61) were commonly shared by accessions collected from Salem, Wyanad and Andaman. EST1[1](0.08 showed the common presence in the accessions collected from Tenkasi, Salem, Wyanad and Andaman. EST5[1] and 81 were observed only in the accession collected from Tenkasi. EST4[2](0.34) and 9[1](0.81) were restricted to the accessions collected from Tiruenlveli, Nagercoil and Kolli Hills (Table 1, Figure 1 B and E).

3.4. Alkaline phosphatase (AKP)

In the alkaline phosphatase enzyme system, only one region (AKP6) of activity with single band (AKP6[1]- 0.51) was observed. In this enzyme system, all the selected accession showed their expression (Table 1, Figure 1 C and F).

The isoenzyme profile emphasized pairing affinity or similarity index analysis of *O. sanctum* is shown in Table 2. A combined cladogram of isoprofile systems was constructed by amalgamating the isoenzymes profiles (Table 1 and 2). This hybrid system reveals the similarity and variation among the *O. sanctum* (Table 2; Figure 2). Highest percentage (0.666 7) of similarity was observed between the accessions collected from Tirunelveli and Kolli Hills, Nagercoil and Kolli Hills and Salem and Andaman while the highest percentage (0.800 0) of variation was observed between the accessions collected from Salem and Andaman. The isozyme profile based cladogram showed two clusters, cluster (C1) included the accessions collected from Tenkasi, Tirunelveli, Nagercoil and Kolli Hills and they showed highest percentage of divergence with the other three accessions. The cluster 2 (C2) included the accessions collected from Wyanad, Salem and Andaman. The cluster 1 was further divided into two branches ($C_1N^1B_1$ and $C_1N^1B_2$) (Figure 2). The

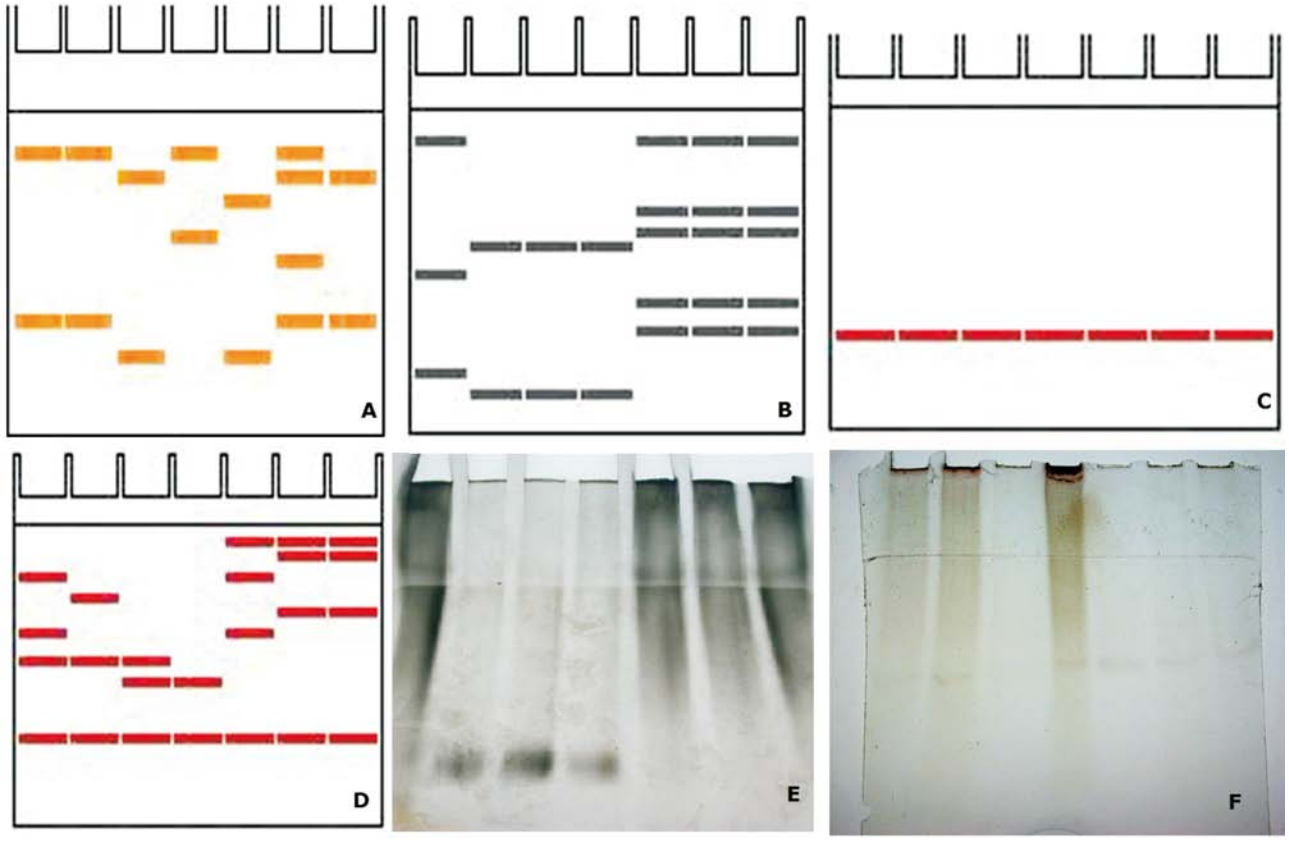

Figure 1. Isozymic profile of the multipotent medicinal plant *O. sanctum* Linn.
A – Zymogram of isoperoxidase of *O. sanctum* L.
B – Zymogram of isoesterase of *O. sanctum* L.
C – Zymogram of alkaline phosphatase of *O. sanctum* L.
D – Zymogram of acid phosphatase of *O. sanctum* L
E – Isoesterase banding pattern of *O. sanctum* L
F – Alkaline Phosphatase banding pattern of *O. sanctum* L

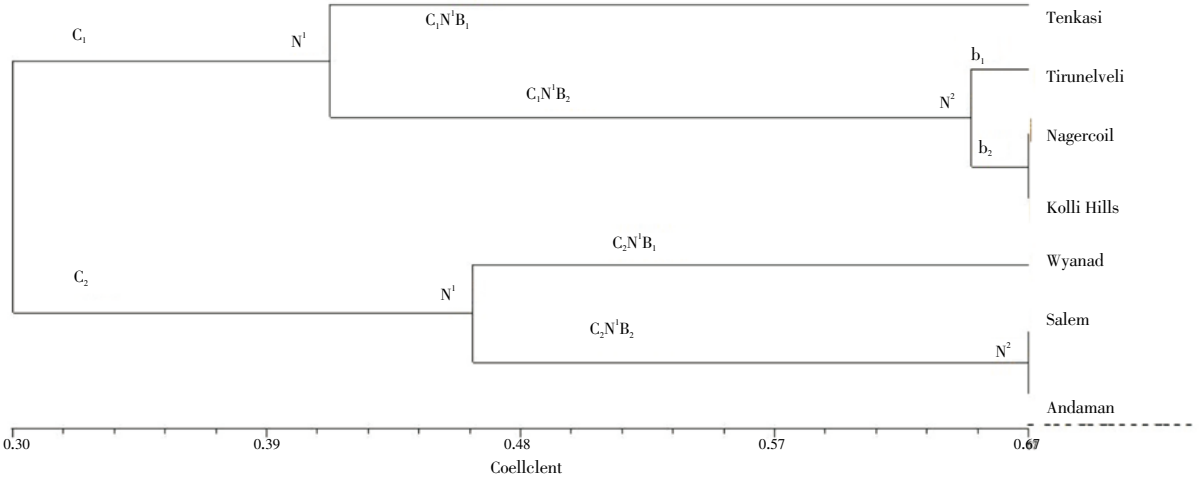

Figure 2. Isozymic profile based cladogram of the multipotent medicinal plant *O. sanctum* Linn.

$C^1N_1B^1$ included only the accessions collected from Tenkasi, the other three accessions *viz*., Tirunelveli, Nagercoil and Kolli Hills were grouped in branch $C_1N^1B_2$. Similar to cluster 1, cluster 2 was also further branched into $C_2N^1B_1$ and $C_2N^1B_2$. The branch $C_2N^1B_1$ included only the accessions collected from Wyanad, the other two accessions were grouped into $C_2N^1B_2$.

4. Discussion

The patterns of genetic variation within and among populations are of interest to diverse fields in plant biology including population genetics, systematics, and conservation. One of the main goals of conservation geneticists is to quantify levels of genetic diversity, as well as the distribution of genetic variability within and between populations, since preservation of the evolutionary potential of endangered species is a primary aim in species conservation[32,33]. Knowledge of the genetic structure of the species will give us information about historical and contemporary patterns of gene flow among populations. Knowledge of genetic variability and its structure will provide a basis for the sustainable management and conservation of populations in threatened plants. For the past four decades, to assess the genetic variation within and between population and species of plants following the demonstration of the utility of enzyme electrophoresis, there has been an ever-increasing use of various types of molecular markers to assess genetic variation[34,35]. However, as the science has progressed, considerations of improved efficiency and sensitivity have promoted the development of new molecular markers, many of which present significant analytical challenges to accurately assessing genetic variation[36]. For more than four decades, isozyme markers have been an invaluable tool for studies of evolutionary genetics, providing plant biologists with a straightforward, low cost means of estimating levels of intra-specific genetic variation[37]. In the present study also the similarity and variation among the various accession of *O. sanctum* are revealed by the isoperoxidase, isoesterase, acid phosphatase and alkaline phosphatase profile. Since 1930, electrophoresis joined with the zymogram technique has been the tool of choice for studies of heritable variation by geneticists, systematists and population biologists. Among several efficient methods for revealing genetic variability within and among plant populations, some of the most widely applied is isozyme electrophoresis[38]. The present study observation is directly coincided with the Hamrick and Godt observations. In electrophoresis, each zone is occupied by a particular isozyme in the form of band and is representative of the appearance of a particular gene locus coding for that isozyme. In certain enzyme system, more than one distinct band could be resolved in a particular zone. These bands could represent allelic isozymes, coded by different alleles of the same gene at locus and thus occupy that particular zone on the gel[27]. In the present study also the similar kind of banding profiles are observed in all enzyme systems indicating the presence of multiple alleles. Isozymes such as esterase and peroxidase have been utilized to assess the genetic similarity and differences at the various taxonomic levels[22-25]. Similarly in the present study, these isozymes are also used as biochemical marker for the systematic study of *O. sanctum*. Unique banding profiles of esterase, peroxidase, acid phosphatase and alkaline phospahatase are observed in *O. sanctum*, which represent the fingerprint of medicinally important plant. Such finger printing is useful in differentiating the species and act as biochemical markers for these species in plant systematic studies.

Conflict of interest statement

We declare that we have no conflict of interest.

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