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GENETIC VARIATION IN THE LIVING REPOSITORY OF *OCIMUM* GERMPLASM

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ABSTRACT:-In the present study different species of *Ocimum* (*Ocimum basilicum and O. sanctum*) were collected from different agro-ecological zones of the world and compared for their phenotypic (qualitative and quantitative) and biochemical trait. Comparatively high variation was seen for seed germination, canopy and spikes/plant, florets/spike and leaf area. A UPGMA cluster, grouped the 9 *Ocimum* genotypes into two major clusters on the basis of total seed protein and phenotypic characters, except Siam Queen and Lime basil stand alone, respectively. Euclidian distance for morphological traits ranged from 3.60 to 7.26. Conversely on the basis of total seed proteins genetic distances ranged from 0.11 to 1.00. Due to greater genetic diversity in *Ocimum* germplasm and its suitability for commercial cultivation even in the area under small holdings, the investigation suggest its genetic as well as biochemical investigation for the production of commercial varieties and exploitation of the plant for economic benefits of the local communities.

Key Words: Ocimum; Diversity; Seed Protein; Morphology; Pakistan.

INTRODUCTION

The genus *Ocimum* is the most popular herbs in the world. It belongs to the Family Lamiaceae, sub family Ocimoideae and includes over 150 different species of herbs and shrubs distributed in tropical regions of Asia, Africa, Central and South America (Darrah, 1980; Vieira and Fontes, 1999; Paton et al., 1999; Simon et al., 1999). The genus *Ocimum*, has long been acclaimed for its diversity (James et al., 1999). Basil is often referred as the "King of the herbs, being widely utilized due to its economic, nutritional, industrial and medicinal importance (Simpson and Conner, 1986; Simon et al., 1990; Khosla, 1995; Javanmardi et al., 2002; 2003; Carovic et al., 2007). Extracts of the plant are used in traditional medicines, and have been shown to contain biologically active constituents that are insecticidal, nematicidal, fungistatic, or antimicrobial (Simon, 1990; Albuquerque 1996). Most commercial basil cultivars available in the market belong to the species *O. basilicum*. *O. basilicum* populations contains high methylcinnamate, methylchavicol, linalool, flavonol-glycosides and rosmarinic acid was present in both flower and leaf tissues (Vieira and Fontes, 1999; Javanmardi et al., 2002; Kosar et al., 2005; Nguyen and Niemeyer, 2008). Lee and Scagel (2009) reported the presence of chicoric acid in basil leaves. Chicoric acid itself has been reported to inhibit HIV integrase (Charvat et al., 2006) and to exhibit antioxidant activities (Dalby-Brown et al., 2005). As dietary supplements, basil represents a more readily available and inexpensive source of these compounds.

Darrah (1974, 1980) classified the *O. basilicum* cultivars in seven types: tall slender types (sweet basil); large-leafed (Italian basil); dwarf types (Bush basil); compact types (Thai basil); *O. basilcum* var. *purpurascens* (purple-colored basil types with sweet basil flavor); purple types (dark opal) which has lobed-leaves with a sweet basil plus clove-like aroma; and *O. citriodorum* types (lemon-flavored basils). In addition, the taxonomy of

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basil is complicated by the existence of numerous botanical varieties, cultivar names, and chemotypes within the species that may not differ significantly in morphology (Simon et al., 1990). However, interspecific hybridization and polyploidy, common occurrences within *Ocimum* genus and have created taxonomic confusion making it difficult to understand the genetic relationship between many basils (Harley et al., 1992; Grayer et al., 1996). The morphological diversity within basil has been accenturated by centuries of cultivation with great variation in pigmentation, leaf shape and size (Anon, 1980).

The present study was aimed to investigate genetic diversity in *Ocimum* populations as inferred by variations in their morphology and electrophoretic profiles of seed proteins as a necessary step for best conservation of *Ocimum* gene pool for future improvement of crop characteristics.

MATERIALS AND METHODS

Plant Material

Total nine basil varieties were selected for the present study. Amongst them, Holy Basil-Thai, Hot wave basil, Italian basil and Purple basil were from Thailand. Whereas, Holy Basil –Local, Garnet basil, Lime basil and Sweet basil were from Pakistan, Russia, UK and Japan, respectively. In the present study, basil germplasm were evaluated for both qualitative and quantitative traits at National Agricultural Research Centre, Islamabad, Pakistan. Qualitative traits under consideration were shape of leaf blade, margin, apex and base, leaf color, venation, odor, stem color and flower color. Quantitative characters included germination %, plant height (cm), canopy (cm), spikes/plant, florets/spike, leaf area (cm^2), and thickness of leaf (mm), thickness of stem (mm) and petiole length (cm) were recorded in the field. Each basil variety was planted in 3mx3m plot using augmented (RCBD) design. Seedlings were planted at 60cm apart with row to row distance of one meter. Data were recorded on three randomly selected plants for all characters.

Protein Extraction, Gel Electrophoresis and Staining

Total seed protein contents were measured with the help of SDS-PAGE. Seeds were grinded to fine powder with the help of mortar and pestle. Then added sample buffer (400µl) to a 0.02 g of fine seed powder as extraction liquid and bromophenol blue (BPB) as tracking dye to follow the movement of protein in the gel. The active ingredients used for the extraction of protein buffer contained 0.5 M Tris-HCl (pH 8.0), 0.2% SDS, 5 M urea and 1% 2-mercaptoethanol. The samples were mixed thoroughly by vortex and centrifuged at 15,000 rpm for 5 min at room temperature (RT). After centrifuging, the crude proteins were recovered as clear supernatant on the top of the tube. Then the supernatant were transferred into new 1.5 ml eppendorf tubes and were stored at -20°C until gel electrophoresis.

The resultant gels were stained with 0.2% (w/v) coomassie brilliant blue (CBB) R250 dissolved in a solution containing 10% (v/v) acetic acid, 40% (v/v) methanol and water in the ratio of 10:40:60 (v/v) for an hour. The gels were then destained by washing with a solution containing 5% (v/v) acetic acid, 20% (v/v) methanol and water in the ratio of 5:20:75 (v/v). Later, gels were dried, using Gel Drying Processor for about two hours.

Data Analysis

Similarity index was used for the presence or absence of polypeptide bands. Presence of the band was scored 1, while absence of the band was marked 0. Data on the presence and absence of the bands were subjected to binary data matrix. Based on the results of electrophoretic band spectra, similarity index (S) was calculated for all possible pairs of protein type electrophoregrams following Sneath and Sokal (1973).

$$S = W/(A + B - W)$$

where,

W = Number of bands of common mobility;

A= number of bands in protein type 'A';

B= number of bands in protein type 'B'.

For similarity matrix, the generated similarity matrix was then converted into a dissimilarity matrix (dissimilarity = 1 - similarity) and used to construct dendrogram by unweighted pair group method with arithmetic averages (Sneath and Sokal, 1973). All the analyses were carried out using statistical package NTSYS-pc, version 2.1 (Applied Biostatistics Inc., USA).

Statistically data were analyzed by using Mstat-C and Excel. Analysis of Variance and Duncan Multiple Range Test (DMRT) was applied for comparison among different collections of *Ocimum*. Unweighted pair group method with an arthimetic average (UPGMA) cluster analysis was used to infer genetic relationships and phylogeny among 9 genotypes of *Ocimum* . All computations were done by using the NTSYS-pc, Version 2.2 package (Rolf, 2005; Rabbani et al., 2008).

RESULTS AND DISCUSSION

In the present study, 9 basil genotypes acquired from Thailand, Japan, Russia, UK and Pakistan were studied for variations in their morphology and total seed proteins. Genetic variation and taxonomic relationships in the genus *Ocimum* were previously investigated using, morphological, chemical, cytological and molecular traits (Khosla, 1995; Grayer et al., 1996; Paton and Putievesky, 1996). Morphological studies of *Ocimum* species showed a high level of variability in recorded traits (Javanmardi et al., 2002). During the study period, data were recorded at different growth stages from seedling to seed maturity stages. Analysis of variance regarding the phenotypic traits seed germination (%), plant height (cm), canopy (cm), spikes / plant, florets / spike, leaf area (cm^2), and thickness of leaf (mm) thickness of stem (mm) and petiole length (cm), showed significant interaction among all the cultivars LSD at

Table 1. Mean performance of Basil lines for variant quantitative traits

Basil varieties	Germination %	Plant height (cm)	Canopy (cm)	Spikes/ plant	Florets/ spike	Leaf area (cm^2)	Thickness of leaf (mm)	Thickness of stem (mm)	Petiole length (cm)
Holy Basil (Local)	17.333c	62cd	223.33b	68.33ab	28d	10.717bc	0.22cd	4.117a	1.167cd
Holy Basil (Thai)	20c	69.66bc	203.33b	48.33bcd	35.33d	8c	0.23cd	2.44a	0.9de
Sweet Basil	36abc	75.66abc	230b	61.33abc	79.33b	15b	0.287bc	5.15a	1de
Siam Queen	50.33a	81ab	343.33a	31.33d	32d	9.4667c	0.34b	4.657a	2.967a
Garnet Basil	11c	65cd	193.33b	60abc	71b	8c	0.293bc	4.463a	1.2cd
Italian Basil	46.66ab	80ab	220b	46bcd	52.667c	34.3a	0.447a	5.137a	2.5b
Basil Lime	60a	55d	350a	76.667a	70b	10bc	0.143d	4.1a	0.6333e
Hotwave Basil	24.66bc	72abc	326.66a	43.333cd	73b	8.7333c	0.213cd	4.94a	1.6c
Purple Basil	16c	85.33a	203.33b	55.667abc	90.667a	7.35c	0.207cd	4.713a	1.067de
Variety mean square	921.4**	292.5**	12734.6**	574.75**	1562.7**	218.51**	0.024 **	2.09 ns	1.183**
LSD at 5%	23.22	12.64	49.11	21.7	10.9	4.811	0.0948	2.554	0.4644
CV (%)	42.82	10.18	11.14	22.98	10.46	22.42	21.73	33.45	18.52

Means followed by same letter (s) do not differ significanlty at 0.5% probability level

Table 2. Summary of statistics of basil germplasm

Descriptors	Mean ± SE	Minimum	Maximum	S.D.	CV (%)
Germination%	31.33 ±3.88	5	70	20.172	64.37
Plant height (cm)	71.741±2.210	50	90	11.481	16.00
Canopy (cm)	254.815±12.792	180	400	66.47	26.08
Spikes / plant	54.556±3.218	25	90	16.722	30.65
Florets / spike	59.111±4.341	26	97	22.555	38.15
Leaf area (cm^2)	12.400±1.693	6	40	8.519	68.69
Thickness of leaf (mm)	0.264±0.019	0.12	0.53	0.100	7.74
Thickness of stem (mm)	4.413±0.321	1.82	7.09	1.667	37.78
Petiole length (cm)	1.448±0.15	0.5	3.1	0.781	53.911

5% (Table 1). Using the morphological data in studying genetic diversity and taxonomic relationships of plants previously scored for many plants (Bult and Kiang, 1992; Zviniene and Pank, 1996). Present study have shown that all the basil varieties significantly differed in germination, plant height, canopy, spikes/plant, florets/spike, leaf area, thickness of leaf, thickness of stem and petiole length (Table 2).

The leaf area recorded by Kritikar and Basu (1984) and Ahmad and Khaliq (2002) ranged from 4 to16cm^2. The mean height of Sweet basil was 46±2.6 though Genovese basil has 49.16±1.9; leaf breadth was reported 3.79±0.2 and 3.71±0.08, respectively (Nazim et al., 2009). The morphological diversity within basil species has been accentuated by centuries of cultivation and is characterized by great variation in pigmentation and leaf shape and size (Simon et al., 1990). During the present study comparatively high variation has been exhibited by seed germination, canopy and spike/plant, florets/spike and leaf area. While others showed low degree of variation. Morphological characters of four genotypes (IO_{S1}, IO_{S2}, IO_{S3}, IO_{S4}) of *Ocimum sanctum* were studied by comparing mean values of some morphological traits (leaf area, number of racemes/plant, number of flowers/raceme, plant height, 1000 seeds weight and days to maturity) to estimate the biological diversity among different genotypes/ land races raised under same climatic condition that showed significant differences with great diversity among *Ocimum* genotypes (Ahmad and Khaliq, 2002). Other studies of Javanmardi et al. (2002) that examined 23 basil accessions (*Ocimum basilicum*) which showed variability in phenotypic characteristics including shape, margin, color and surface of leaf stem and flower color, plant height and inflorescence length. Basil plants have square stems, fragrant opposite leaves, and whorled flowers on spiked inflorescences (Darrah, 1980).

Qualitative traits showed a wide range of variation. seven varieties were found to be green and two deep purple (garnet and purple basil). The flower color of Siam Queen, Holy basil-Thai and local were lilac, garnet basil was purple and other four varieties Italian basil, Lime basil, Hot wave basil and purple basil showed white inflorescence (Figure 1). Leaf odor of most of the varieties was clove like except lime basil that exhibit lemon like odor. Observations for leaf blade were found ovate for most of the basil varieties except Holy basil-Thai and Sweet basil which has elliptic leaf blade. However Siam Queen was lanceolate. No variation was observed for leaf apex, venation and margin. However, variation was observed in leaf base as shown in Holy basil –Thai, Hot wave basil and purple basil that were found obtuse and the other were cuneate. Morphological differences was reported by Nazim et al. (2009) for Sweet basil that exhibit green leaf color while Genovese basil acquires purplish white. There is also dissimilarity in their leaf margin. Sweet basil has slightly undulate leaf margin whereas basil has serrate type. Both species are also different in color of

Table 3. Genetic distance (above bold) and Euclidean distance (below bold) between basil varieties

Basil-Variety	HB(L)	HB(T)	SB	SQ	GB	IB	LB	HB	PB
Holy-Basil(local)	**0.00**	0.12	0.12	0.80	0.43	0.11	0.18	0.38	0.06
Holy-Basil(Thai)	3.81	**0.00**	0.00	1.00	0.67	0.25	0.33	0.64	0.07
Sweet-Basil	4.61	4.60	**0.00**	1.00	0.67	0.25	0.33	0.64	0.07
Siam-Queen	6.29	5.42	5.14	**0.00**	0.67	0.80	0.78	0.60	1.00
Garnet-Basil	5.46	5.36	3.98	7.11	**0.00**	0.67	0.80	0.78	0.60
Italian-Basil	5.87	6.10	3.88	5.29	5.58	**0.00**	0.06	0.38	0.18
Lime-Basil	5.61	6.05	5.32	6.87	6.24	7.16	**0.00**	0.50	0.25
Hotwave-Basil	3.97	4.93	3.60	5.23	4.62	5.02	5.36	**0.00**	0.50
Purple-Basil	5.93	5.94	4.70	7.26	3.78	6.36	7.00	4.31	**0.00**

Flowers. Sweet basil has white flower though Genovese basil has purplish white flower, seed color in both varieties is black with oval shape and verticellaster inflorescence. Seeds of both varieties successfully germinated 91-100%. *Ocimum* taxonomy is confused due to interspecific hybridization, polyploidization, and the existence of chemotypes or chemical races with similar morphology (Carovic et al., 2006). Taxonomy of basil *Ocimum* is complicated by inter- and intra-specific hybridization, and the existence of numerous cultivars and chemotypes within species that do not differ significantly in morphology (Vieira and Fontes, 1999).

The phenotypic relationship among the basil genotype was assessed by an Unweighted pair group method with an arthimetic average (UPGMA) cluster analysis of the similarity matrix to infer genetic relationships and phylogeny among genotypes. A UPGMA cluster diagram grouped the 9 genotypes into two major clusters, I and II, differentiating the Siam Queen and Italian basil from other genotypes (Holy basil, Sweet basil, Hotwave basil, Garnet basil and Purple basil). Whereas Lime basil stand alone (Figure 3). The separation of Lime basil from all other is correlated with clear differences in most of the scored morphological characters. Grouping the genotype within clusters reflects a genetic basis of plant form in *Ocimum.* Similar trend of grouping was observed on the basis of total seed proteins using SDS –PAGE that discriminate 9 genotypes into two major clusters. Garnet basil and Hotwave basil fall in one group of Thailand. Other genotypes categorize under group II except Siam Queen that stand alone (Figure 2). Ahmad and Khaliq (2002) work out on total seeds proteins of four genotypes of *Ocimum sanctum* in SDS PAGE created diverse banding pattern among the genotypes compared that were collected from northern Hima-


Sweet-Basil

Italian Basil

Holly Basil-local


Siam Queen Basil


Purple Basil

Basil Lime

Hot Wave Basil


Garnet Basil

Holly Basil-Rama

layan regions of Pakistan. Carovic et al. (2006) determined phylogenetic relationships by neighbour joining cluster analysis based on dice distance between *Ocimum* accessions. Within the *O. basilicum* cluster, similar accessions grouped together specifically *O. minimum, O. basilicum* 'Dark Opal' and *O. basilicum* var. *difforme* accessions. In the present study, the distances (genetic and Euclidian) among basil cultivars above bold and below bold represents the genetic distances on the basis of total seed proteins and morphological traits, respectively (Table 3). Euclidian distance for morphological traits ranged from 3.60 to 7.26 (below bold) among 9 basil cultivars. The lowest distance recorded was 3.60 between Hot wave basil and Sweet basil. Whereas diverse variation accompanied by Purple basil and Siam Queen Basil (7.26). The 2nd highest Euclidian distance in conjunction found with Lime basil and Italian basil (7.11). However, on the basis of total seed proteins genetic distances ranged from 0.11 to 1.00 (Table 3; above bold). Lowest distance recorded 0.06 between Holy basil-Local with Purple basil and Italian basil with Lime basil. At 45% dissimilarity, seven populations of two species of *Ocimum* were grouped into four categories and farthest genetic distance between *Ocimum kilimandscharcum* and *O. basilicum*. Nearest genetic distance were revealed among accessions of sweet basil. The higher genetic polymorphism in seven population of *Ocimum* is dependent on the amount of sexual reproduction, whereas low levels of genetic variation are often associated with asexual propagation (Abd El-Zaher et al., 2006).

The SDS-PAGE techniques have more advantages in the classification of genotypes. The common band shown by all the genotypes (40- 60 KDa) indicated that the genotypes have some common heritage. Abd El-Zaher et al. (2006) reported large variation in the *Ocimum* seed proteins, lie in between 60 and 20 KDa. The large variation in the number of protein bands among the studied *Ocimum* accessions reflects the high level of genetic variation and within *Ocimum* at both specific and infra specific levels (Figure 2).

The constructed trees based on variation in morphological (Figure 3) and seed proteins (Figure 2) clearly demonstrated the

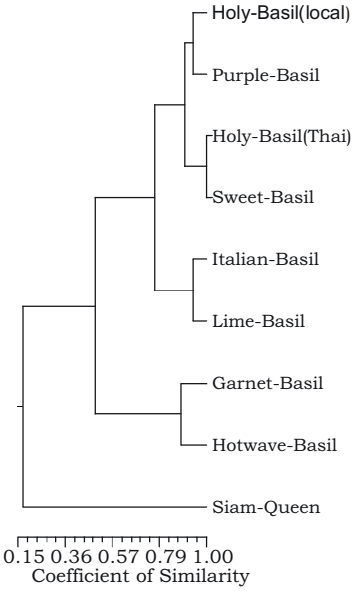

Figure 2. Cluster observed in 9 genotypes of basil on the basis of 10 polymorphic bands of total seed Protein

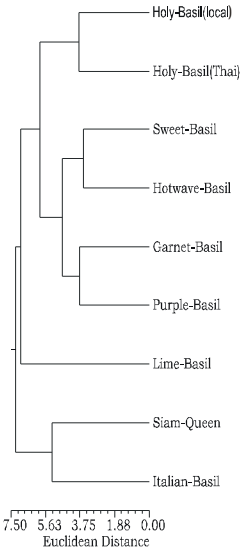

Figure 3. Cluster observed in 9 genotypes of basil on the basis of morphological chracterization

existence of genetic diversity among and within populations of *Ocimum* that might be related to natural hybridization and fluctuations in environmental conditions. Seed proteins and isozyme polymorphism exhibited validity for studying genetic diversity and taxonomic relationships in *Ocimum* at both species and infra-specific levels (Abd El-Zaher et al., 2006).

Electrophoresis of seed proteins was previously used for cultivar identification for many other plants; as *Vicia faba* (Stegmann et al., 1980) and *Linum usitatissimum* (Sammour, 1988). The technique had been used utilized by Masood et al. (1995) in different genotypes of wheat, Irfan (2000) in *Adhatoda vesica*, Ahmad and Kamal (2002) in *Hyppophae rhamnides*.

The variable banding pattern shown by Siam Queen Basil made it different from other genotypes. As proteins are the translational products of genes therefore, the differences in proteins could be related with such differences in genes responsible for synthesis of these proteins. The molecular techniques including SDS-PAGE are very strong to determine among the biological organisms based on such molecular markers. Hence are very common to investigate the genetic diversity among the species, varieties and genotypes (Irfan 2000; Ahmad and Kamal, 2002).

Estimating the genetic diversity levels both within and among *Ocimum* populations of a crop is necessary for the best conservation of its gene pool. The diversity in basil based on appearance, flavors, fragrances and natural pigments offers a wealth of opportunities for developing new culinary, ornamental, and industrial crops and genetic improvement through selection and hybridization of diverse genotypes. The variability provides a base for future improvement of plant type through conventional and non conventional means therefore, will have great value in breeding commercial varieties of *Ocimum*.

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