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Morpho-molecular Variability and Heritability in *Ocimum sanctum* Genotypes from Northern Himalayan Regions of Pakistan

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Abstract: *Ocimum sanctum* has immense medicinal value against malaria, gastric diseases, blood and heart diseases, cough, bronchitis, asthma, chronic fever, liver disorder, earache, ringworm and skin diseases. In order to evaluate the genetic diversity among different genotypes of *Ocimum* for the development of better varieties, plant characteristics including phenotypic and genotypic variation as well as biochemical analysis were conducted. The seeds of four genotypes, collected from different localities of District Poonch, Azad Kashmir were sown in pots and transplanted to the field. The morphological studies based on leaf area, number of racemes/plant, number of flowers/raceme, plant height, 1000-seeds weight and days to maturity indicated large diversity among genotypes. Total seed proteins in SDS-PAGE also produced diverse banding pattern among the genotypes compared. Due to the larger genetic diversity in germplasm of *Ocimum sanctum* and its suitability for commercial cultivation in the area under small land holdings, the investigation suggests it's genetic as well as biochemical investigation on larger scale for the production of commercial varieties and exploitation of the plant for economic benefits of the local communities.

Key words: *Ocimum sanctum*, genotypes, medicinal value, genetic diversity, SDS-PAGE, seed proteins

Introduction

Ocimum sanctum belongs to the family Labiateae, Order Lamiales class Magnoliopsida (Watson and Dallwitz, 1999) is one of the valuable herbs of this area with inherent ability to grow under natural conditions. It is common medicinal herb found in wild as well as under cultivation were in Azad Jammu and Kashmir. Locally known as 'Babri', Holy Basil, Tulsi, and Bastlic saint in English, Urdu, Greece and French respectively. The multipurpose plant grows in wide variety of soils and climatic conditions requiring long days and relatively high temperature for good growth and high yield of essential oils. This plant is cultivated normally in kitchen gardens for culinary purposes. The plant is distributed throughout India, Pakistan and Kashmir ascending up to 1800 m in Himalayas and down to the tropical areas, Malaya, Australia, Philippines, Brazil, Western Asia, Arabia, Persia, Nepal and Egypt (MGrieve, 1992; Bhattacharjee, 1998). The major constituents of the plant are volatile oils including Euginol, Carvacrol, Methyl-euginol, β-Caryophyllene, Mithyle-Charvicol, β-elemene, β-Ocimine, a-humulene, Germacrene-D, β-bisabolene, α-bisaboleme. Besides the volatile oils the plant is reported to contain alkaloids, flavonoids, glycosides, saponins, tannins, triterpene, and ascorbic acid and carotene l. (Baquar, 1989; Pino *et al.*, 1998; Raju *et al.*, 1999; Sammbamurty and Subrahmanyan, 2000).

Plant extracts are used as pesticides against different pests of plants like insects, viruses and fungi. Plant and leaf extracts of *O. sanctum* have strong anti fungal activity against *Pyricularia oryzae*, Drechslera oryzae and *Corticium sasakii* (Tewari and Premalatha-Dath, 1984). The influence of spore germination of fungi, has been used to control styler – end rot and soft rot of grapes caused by *Phomopsis psidii* and *P. viticola* at a concentration of 75% *in vitro* (Arya, 1988) that effectively reduce the radial *in vitro* growth of *Pyricularia oryzae, Cochiliobotus miyabeanus* and *Rhizoctonia solanai* and help to control brown spot, blast and sheath blight diseases in rice *in vivo* (Tewari and Nayk, 1991). It can also be used against fruit rot fungi, soft rot of spong ground fruit caused by *Fusarium scirpi* (Ahmad and Parsad, 1995), as well as phomopsis blight and fruit rot in brinjal (Panda and Tripathy, 1996). It is found to be most effective against spore germination of *Alternarie brassicai* the causal agent of *Alternaria* blight of mustard (Ram, 1997). Essential oils of *O. sanctum* have antifungal activity against *Aspergillus flavus, A. fumigatus, A. parasiticus, Helmenthosporium oxysporum* and *Trichoderma viride* (Prakash and Singh, 1986).

Medicinally the plant is useful in a variety of diseases in humans, animals and experimentally in laboratory animals. The infusion of leaves of *Ocimum sanctum* is given in malaria, as a stomachic in gastric diseases of children and hepatic affections; leaf juice in paste with metallic preparations are liked up; mixed with lime juice are used in ringworm; poured into ear to control earache; to control chronic fever, hemorrhage, dysentery and dyspepsia, colic in children, catarrhal fever, cold stages of intermittent fever; check vomiting and destroy intestinal worms, used as expectorant in cough and bronchitis, cough and infections of chest, antidote in snake bites, bronchitis and diarrhoea, seminal debility, ringworm and skin diseases, earache, gastric disease, malarial fever and insect bites (Dastur, 1988; Baquar, 1989; Bhattacharjee, 1998). Ethanolic extracts of the plant inhibit the activity of polio virus type III up to 99.90% while aqueous extracts to 99.68% (Parida *et al.*, 1997). Extracts have been used for elimination of bacterial contaminants of raw drinking water obtained directly from the sources to reduce water born diseases (Kumar and Gopal, 1999). Leaf extracts of *Ocimum sanctum* have antitumour activity against skin papillomagenesis (Tumor) in mice (Prashar *et al.*, 1994), antiulcerogenic effect in rats (Vanisaree and Devaki, 1995), chemopreventive of chemical carcinogenesis in different animal model system (Banerjee *et al.*, 1996), antitoxic effect against the toxicity caused by $CuSO_4$ in rats (Shyamala and Devaki, 1996), have radio modulating effect and protect mice against radiation lethality (Ganasoundari *et al.*, 1997), possess potent antioxidant activities (Maulik *et al.*, 1997), have *in vivo* protection against cytogenetic damage in Swiss mouse. (Ganasoundari *et al.*, 1997), regulate thyroid function and possess anti oxidative activities in experimental male mouse (Panda and Kar, 1998). Fixed oil of *Ocimum sanctum* has anti arthritic as well as anti edematic activities in laboratory animals (Singh and Majundar, 1996), possess anti-inflammatory activities and used to control castor oil induced diarrhoea in mouse (Singh *et al.*, 1996). Two flavonoides of *Ocimum sanctum* (Orienting and Vicenin) are promising for human radiation protection (Devi *et al.*, 1998). *Ocimum sauctum* has immuno potentiating effects in the form of dry leaf powder on cell medicated immune response in poultry, naturally infested with IBD virus (Anonymous, 1998), and Humoral immune response in poultry (Anonymous, 1998). It also has antibacterial activity against fish and shrimp pathogenic bacteria (Anonymous, 1998).

Considering the importance of *Ocimum sanctum* and its abundance in Azad Jammu and Kashmir the studies were initiated to classify its various land races and genotypes for its commercial exploitation. Due to the advent of molecular techniques the variability among different genotypes/land races as well as

populations could be determined in short period of time and with greater authenticity. The various techniques based on DNA and protein, which include RFLP's, AFLP's, RAPD's micro satellite DNA finger printing and SDS-PAGE. SDS-PAGE technique was utilized for the determination of the variability in different genotypes of *Ocimum sanctum* from various regions of district Poonch. The genetic improvement of any crop depends upon the existence of initial variability for rational genetic improvement through selection and hybridization of diverse genotypes.

It is to be noted that the lands holding in Azad Kashmir are small therefore; the cereal crops cannot be cultivated economically. However the valuable cash crops like *Ocimum sanctum* have the potential for economic cultivation and sustenance of the local communities. The specific objectives of the study were:

1) To collect, evaluate and cultivate the local genotypes of *Ocimum sanctum* for their commercial potential.
2) To evaluate the variability among different genotypes using morpho-molecular techniques for conservation and future improvement programme.

Materials and Methods

Study area: Azad Jammu and Kashmir, a Himalayan region of Pakistan lies between longitude 73 – 75° and latitude 33 – 36° mainly hilly and sub mountainous with valleys and stretches of the plains. Annual average rainfall is about 1560-mm with mild summer and very cold and snowy winter. The topographic nature of the area does not suit for the production of cereal crops because of the small land holdings and fragmented steep hills. These steep hills are mostly covered with conifer forests enriched with large diversity of medicinal plants, wild life, soil micro flora and fauna of great value (Ahmad *et al.*, 1998).

The study includes different genotypes of *Ocimum sanctum*, which were tested both on molecular level and morphologically for their genetic diversity and yield-related traits. Seeds of *Ocimum sanctum* from the four localities, Tatrinot, Dothan, Pothi and Khaigala collected, multiplied and stored under cold at the P.B.G. laboratory of University College of Agriculture Rawalakot were sown in pots to raise a nursery for each representative locality. Four genotypes were named as IO_{S1}, IO_{S2}, IO_{S3} and IO_{S4}. A randomized complete block design (RCBD) was followed for transplanting the nursery plants in field in three replicates. For morphological studies of different genotypes, 5 plants were selected randomly from each replication and were properly tagged to compare: leaf area, number of racemes/plant, number of flowers/raceme, plant height, 1000 seeds weight, duration of plants from sowing to transplanting and seed maturity.

The mean values of five observations/block for each trait were analyzed using the standard method of Steel and Torrie (1980) and Duncan's multiple range test. The genetic components of variance were calculated as outlined by Johnson *et al.* (1956). The estimates of broad sense variability (h^2) genetic advance (ΔG) at 5% selection intensity ($K = 2.06$) and relative genetic advance (REGA) were obtained by using procedures of Allard (1960).

Biochemical/Molecular comparisons among the genotypes were performed using total seed proteins and standard SDS-PAGE techniques (Ahmad and Kamal, 2002). Preparation and polymerization of gels and electrophoresis was carried out by a little modification in the standard method of Laemmli (1970). Photograph of the gels were taken after staining and destaining, for reference. The distance covered by different protein bands were closely observed on a light box, the pattern was also drawn on a paper for later reference. The comparisons were made between the common and variable banding pattern for genetic diversity and evolutionary relationship among genotypes.

Results and Discussion

Morphological studies: Morphological characters were compared to estimate the biological diversity among different genotypes/land races raised under same climatic conditions and applying same cultural practices. For the estimation of genetic variability among them, mean values of some morphological traits are compared (Table 1). Phenotypic and Genotypic variance (δp^2, δg^2) and covariance (PCV, GCV), haritability (h^2), genetic advance (ΔG) and relative genetic advance (REGA) in different land races of *Ocimum sanctum* are compared (Table 2). Analysis of variance for different characters compared among four genotypes of *Ocimum sanctum* is also presented (Table 3).

The comparison showed that the large variability seemed to be more genetic rather than environmental. Great variability occurred for morphological characters i.e. leaf area, number of racemes/plant, number of flowers/raceme, plant height, 1000 seeds weight and days to maturity. Mean values of leaf area showed variability in the order of $IOs_4 > IOs_1 > IOs_3 > IOs_2$ (Table 1). Higher values of heritability showed lesser environmental and greater genetic effects. Highest values of genotypic and phenotypic covariance indicated wide range of variability and high heritability associated with higher values of relative genetic advance. It would employ that additive gene effects were more important (Table 2). The leaf area recorded by Kritikar and Basu (1984) ranged from 4-16 cm^2. This finding is in accordance with the results of present study.

Values for number of racemes/plant were in the order of $IOs_4 > IOs_3 > IOs_1 > IOs_2$ which indicated variability among the genotypes for this character (Table 1). Highest values for heritability (Table 2) indicated that there was more genetic and less environmental influence. Higher heritability in association with genetic advance showed that additive gene effects were more important for this trait as well. Variability was also found in the number of flowers/raceme as $IOs_4 > IOs_3 > IOs_1 > IOs_2$ (Table 1). Higher heritability revealed less environmental and greater genetic effects on four genotypes/land races, while values of covariance, genetic advance and relative genetic advance showed that range of variability was low. Such indications may be due to non-additive gene effects. It means that the character had less range of variability, however the environmental effects were less than the genetic effects (Table 2).

The comparison of values for plant height had also shown variability in the order of $IOs_4 > IOs_2 > IOs_3 > IOs_1$ (Table 2), indicating more genetic influence on the four genotypes, wide range of genetic variability and additive gene effects for the character. The average plant height recorded by Kritikar and Basu (1984), ranged from 30-60 cm and was also comparable with the results of present investigation. The genotypes/land races were also diverse in respect of 1000-seeds weight. The order of variability was $IOs_4 > IOs_3 > IOs_2 > IOs_1$. The values of heritability and genotypic and phenotypic covariance showed wider range of variability and more genetic influence. Low values of genetic advance indicated non-additive gene effect. Days to maturity for genotypes were also variable in the order of $IOs_4 > IOs_3 > IOs_2 > IOs_1$.

The detailed morphological results highlighted the significant differences in genotypes compared (Table 3). As the genotype/land races were adapted to different climatic conditions therefore the natural selection had played its role and resulted in the biological diversity of *Ocimum sanctum* in Azad Kashmir. Considering the genetic variability among genotypes of *Ocimum sanctum*, its medicinal importance (Kumari *et al.*, 1994; Agarwal *et al.*, 1990; Sing and Majumdar, 1996; Parida *et al.*, 1997; Gana Soundari *et al.*, 1997; Malik *et al.*, 1997; Kelm and Nair, 1998; Bhattacharjee, 1998; Devi *et al.*, 1998; Kumar and Gopal, 1999; Sammbamurty and Subrahmanyan, 2000) and insecticidal as well as pesticidal uses (Satpathi and Ghatak, 1990; Tewari and Naik, 1991; Ahmad and Parsad, 1995; Panda and Tripathy, 1996; Ram, 1997) may be of great value. Because genes are responsible to direct the synthesis of different bio-chemicals therefore, the variable genotypes may be important for variable bio-chemicals too. 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 *O. sanctum*.

Table 1: Mean value of some morphological traits in four genotypes of *Ocimum sanctum*

Traits	IOs_1	IOs_2	IOs_3	IOs_4
Leaf area (cm^2)	10.15	8.47	9.69	11.89
Number of racemes/plant	143.93	116.43	147.1	180.67
Number of flowers/raceme	64.29	62.60	66.47	75.27
Plant height (cm)	43.20	47.30	45.90	56.10
1000-seeds weight (g)	1.06	1.06	1.44	1.47
Days to maturity	148.00	154.00	157.00	167.00

Table 2: Phenotypic and genotypic variance and covariance, haritability, genetic advance and relative genetic advance in four land races of *Ocimum sanctum*

	Leaf area	No. of racemes/plot	No. of flowers/raceme	Plant height	1000 grains weight
δp^2	2.12	731.29	31.75	31.71	0.05
δg^2	1.81	630.36	25.38	27.08	0.043
PCV (%)	14.46	18.68	8.30	11.68	17.75
GCV (%)	13.36	7.36	7.50	10.79	16.64
h^2(%)	85.38	86.19	79.93	85.41	86.00
ΔG	2.56	48.01	9.27	9.90	0.39
REGA (%)	25.42	33.16	13.81	20.54	31.44

δp^2 = genetic variance, δg^2 = phenotypic variance, PCV = phenotypic covariance, GCV = genetic covariance, h2 = heritability, ΔG = genetic advance and REGA = relative genetic advance.

Table 3: Mean squares of various parameters corresponding to genetic variation in *Ocimum sanctum* genotypes

SOV	D.F.	Leaf Area	Number of racemes/plant	Number of flower/raceme	Plant height	1000-grain weight
Blocks	2	0.04	15.68	10.33	0.08	0.03
Genotypes	3	6.35*	193.88*	95.25*	95.12*	0.15*
Error	6	0.91	203.79	19.09	13.78	0.02

*significant at 5% level

Table 4: SDS – PAGE fractionation of seed proteins from different genotypes of *Ocimum sanctum*

Distance (cm)	IOs_1	IOs_2	IOs_3	IOs_4
0.0				
0.5				
1.0		--		--
1.5	--		--	
2.0		--		
2.5	--			
3.0	--			--
3.5		--	--	
4.0				--
4.5	--	--	--	--
5.0				

Molecular studies: There was great variability among the four genotypes of *Ocimum sanctum* on the basis of morphological traits but these differences did not prove directly that the variability was due to genetic differences. Now-a-days advance molecular techniques like DNA protein and RNA analysis are available to determine the exact and direct genetic differences among species and genotypes. The SDS–PAGE techniques having more advantages in the classification of genotypes were used for present investigation (Table 4). The technique had been utilized by Masood *et al*. (1995) in different genotypes of wheat, Irfan (2000) in *Adhatoda vesica*, Ahmad and Kamal (2002) in *Hyppophae rhamnoides*.

The common band shown by all the genotypes (4.5 cm) indicated that the genotypes have some common heritage (Table 4). Another common band shown by IOs_3 and IOs_2 (3.5 cm) revealed their closer relationship. Such relationship has also been observed in their time of maturity. The variable banding pattern shown by IOs_1 made it different from other genotypes. Similar results were observed from morphological studies for IOs_1. The banding pattern of IOs_4 was also in accord with the morphological studies. IOs_4 showed more leaf area, number of racemes/plant, number of flowers/raceme, plant height, 1000 seeds weight and days to maturity and was different from all other genotypes. The presence of common bands i.e. one band in all genotypes and one shared band between IOs_2 and IOs_3 is the indication of their close genetic base. Whereas different banding pattern would indicate their genetic difference. As proteins are the translational products of genes therefore, the difference in proteins could directly be related with such differences in genes responsible for synthesis of these proteins. The molecular techniques including SDS-PAGE are very strong to determine the relationship and differences among the biological organisms based on such molecular markers. Hence are very commonly used to investigate the genetic diversity among the species, varieties and genotypes (Waines and Payne, 1987; Moller and Spoor, 1993; Ciaffi *et al.*, 1993; Masood *et al.*, 1995; Ashour *et al.*, 1995; Irfan, 2000; Ahmad and Kamal, 2002).

The variability observed in *Ocimum sanctum* genotypes on the basis of morpho-molecular investigations indicated the genetic diversity among natural populations/genotypes found in district Poonch, Azad Kashmir. The natural genetic diversity has lots of significance for breeding better varieties of the medicinal plant in the area for its commercial exploitation and a source of sustainable development of agriculture for farmers of the mountain community with scarce land resources. The investigation was limited to one district only but the diversity observed reveals that there may be a larger genetic variation among the natural genotypes of the area. The detailed investigation in this respect may be more meaningful when such plants of greater economic potential are readily available producing natural bioactive products of commercial value.

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