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Effect of Salicylic Acid on Genetic Variation of *Ocimum basilicum* (Basil) Based on ISSR, RAPD and Protein Markers

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Abstract: Essential oils extracted from basil (*Ocimum spp.*) are used as flavor foods and oral products as well as in fragrances and in traditional medicines. The genus *Ocimum* contains around 30 native species to the tropics and subtropics with species cultivated in temperate regions. Results indicated that salicylic acid (SA) affected on aromatic essential oils and flavonoids using principal component analysis. The response of sweet basil plants to foliar application of salicylic acid (SA) at 10^{-5}, 10^{-4} and 10^{-3} m was determined in pot experiments conducted during 2007-2008. In the present work, essential oil composition RAPD, ISSR and protein markers were studied to estimate the effect of salicylic acid with different concentration among *(Ocimum basilicum L).* The essential oil revealed that common components of *Ocimum basilicum* essential oil under all treatments were linalool (46.63 - 43.32%), methyl eugenol (13.83 - 5.68%), 1, 8 - cineol (13.20 - 4.43%), eugenol (12.64-7.16%) and α-cadinol (9.59 - 4.46%). SA at 10^{-4} M increased the production of top quantity and quality of basil oil to the fragrance and food industries by increasing the percentage of eugenol and antioxidant activity in the herb. RAPD marker were used in order to assess the genetic relatedness among the basil cultivars by using four successfully exhibited a total of 29 fragments cross all treatments and control with eight specific markers. A dendrogram represented the phylogenetic relationships grouped all plants into two clusters. ISSR marker using 10 primers showed a total of 67 fragments cross all the treated and control plants. Thirteen specific fragments were detected. Dendrogram trees based on ISSR and combined data of RAPD and ISSR analyses showed two clusters. The electrophoretic analysis of the total proteins showed effect of treatment comparing with control (by disappearance of some bands).

Key words: *Ocimum basilicum,* RAPD, ISSR, SDS-PAGE protein, salicylic acid

INTRODUCTION

The family lamiaceae includes large number of essential oil plants and the most important members are sweet basil (*Ocimum basilicum*). Sweet basil is one of the most wide spread spices in the world and its dried leaves are used commonly for flavoring many food products. Salicylate content of spices was bio-available and may contribute to the low cancer incidence in rural India [1]. Basil contains a wide range of essential oils rich in phenolic compounds [2,3] and polyphenols such as flavonoids and anthocyanins[4]. The volatile basil oil is used in pharmaceutical and perfume industry.

The quality of the product is usually determined by chemical analysis, whereas in food industry applications sensory tests are also practiced. Epidemiological studies have suggested positive associations between the consumption of phenolic-rich foods or beverages and the prevention of disease due to the presence of antioxidant components such as phenolics [5,6].

Salicylic acid (SA) naturally occurs in plants in very low amounts. Phyllis [7] has been found that salicylic acid can stimulate flowering. Moreover, salicylic acid has been found to cause temperature increases of as much as 14°C above the ambient temperature in the Arum group of plants (eg. duckweed, Skunk Cabbage). It has been identified as an important signaling elements involved in establishing the local and systemic disease resistance response of plants after pathogen attack [8]. After a pathogen attack, SA levels often increases and induces the expression of pathogenesis related proteins and initiates the development of systematic acquired resistance and hypersensitive response [9]. Exogenously added SA also increased the heat resistance of mustard [10]. Mohamed *et al.* [11] reported

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that the potential role of salicylic acid for improved survival percentage of plantlets and stress tolerance during acclimatization. Young maize plants exhibited increased cold tolerance upon treatment with SA or aspirin [12]. Exogenously added SA also increased the heat tolerance of mustard [10]. Moreover, SA treatments at 0.5 mM strongly or completely suppressed the Cd-induced up-regulation of the antioxidant enzyme activities of barley [13]. SA has a direct physiological effect through the alteration of antioxidant enzyme activities. SA induces flowering, increase flower life, retards senescence and increases cell metabolic rate.

Recently global interest in oriental medicine, production of those plants has grown even more over the following years. Since many species and varieties exist, development of molecular markers would be important for quality assessment in the medicinal industry [14] and several molecular markers such as RAPD and ISSR have been rapidly integrated into the tools available for genome analysis. Harisaranraj *et al.* [15] suggested that genetic relationships in *Ocimum* species using RAPD analysis are useful for plant improvement and considered as efficient way to conserve genetic resources of *Ocimum* species, in addition to their effective medicinal uses. Singh *et al.* [16] studied the genetic variation of *Ocimum gratissimum* L. based on volatile oil constituents, flavonoids and RAPD markers and De Masi Luigi *et al.* [17] assessed the relationships among *Ocimum basilicum L.* genotypes using morphological characteristics, essential oil composition and RAPD markers. Inter simple sequence repeats (ISSR)-PCR is a method, which involves the use of microsatellite sequences as primers in a polymerase chain reaction to generate molecular markers.

The present study was undertaken to examine the effects of foliar application of SA ($M10^{-5}$, $M10^{-4}$ and $M10^{-3}$) on essential oil content and the genetic plant materials using electrophoresis' patterns of SDS-PAGE protein, random amplified polymorphic DNA (RAPD) and Inter-simple sequence repeat (ISSR) markers.

MATERIALS AND METHODS

Plant Material: A pot experiment was conducted at the experimental farm of Helwan University, Cairo, Egypt during 2007/2008. Seeds of *Ocimum basilicum* L. (kindly provided by Horticultural Research Institute, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt) were sown in beds on 1st Feb. Uniform 45 days old seedlings were transplanted per earthenware pot (40 cm in diameter) of 15 kg of sandy loam soil. The pots were arranged in complete randomized blocks design with four treatments, four replicates per treatment and each replicate represented by 2 plants. Irrigation was regularly carried out at intervals to keep the moisture content of the soil to field capacity. Plants were foliar sprayed twice with SA (10^{-5}, 10^{-4} and 10^{-3} M), the first spray was done after 75 days of sowing, while the second spray was applied one week later after the first one. Control plants were sprayed with distilled water and the volume of the spraying solution was maintained just to cover completely the plants foliage till drip. The plants were collected after 2 months of transplantation.

Essential Oil: Quantitative determination of *O. baslicum,* essential oil from the fresh samples was achieved by hydro-distillation at first, second and third cuts. Essential oils from cut plants were separated and analyzed qualitatively by GC/MS at National Research Centre,Dokki, Cairo. The GC analysis was carried out using Varian-3400 GC equipped with a DB-5 fused silica capillary column. Mass spectrometer was a Varian–Finnigan SSQ 7000.

DNA Extraction, RAPD and ISSR Analysis: Stored leaves were pulverized in liquid nitrogen and DNA was extracted from each treated plant of *Ocimum basilicum L.* according to Doyle and Doyle [18]. RAPD analysis was performed using ten 10-mer random primers (Carl Roth GmbH Co, Germany) as shown in Table (2). RAPD amplification reaction was used in a final volume of 25 µl containing 1X PCR buffer (2 mM $MgCl_2$, 200 mM dNTPs, 10 mM primer, 25 ng of template DNA and 2 µ of *Taq* polymerase (BIOLINE COMPANY). Reactions were performed in Perkin-Elmer 9600 thermocycler (HYBAID PCR EXPRESS). RAPD-PCR was performed according to Williams *et al.* [19] as one cycle of 94°C for 5 min (denaturation), 40 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 90 sec (annealing), extension at 72°C for 2 min and a final extension of 7 min at 72°C. ISSR amplification reaction was used in a total volume of 20 µl containing 30 ng temple DNA, 1X PCR buffer, 200 µM dNTPs, 10 pmol of each primer and 1 unit *Taq* polymerase. ISSR-PCR was performed according to Ratnaparkhe *et al.* [20] as one cycle of 95^0C for 5 min, 35 cycles of 94^0C for 1.5 min, annealing at 55°C for 1.5 min and final extension 72°C for 10 minutes (one cycle) and hold at 4°C. Primer sequences and amplification conditions presented in Table 2 according to Ramsay *et al.* [21]. PCR products

Table 1: Effect of foliar spray with salicylic acid on the composition of essential oil of *Ocimum basilicum* L. plants

		Salicylic acid		
Oil components%	Control	10^{-5} M	10^{-4} M	10^{-3} M
α-Pinene	0.45	0.46	0.05	0.58
Camphene	0.04	0.08	-	-
Sabinene	1.46	1.47	0.21	2.01
α-myrcene	1.3	1.5	0.26	1.7
Limonen	0.05	-	-	-
1,8-cineol	13.2	14.44	4.43	15.31
Ocimene	1.16	1.73	0.54	1.38
γ-terpinene	0.37	0.33	0.29	0.29
Linalool	46.63	45.62	46.63	43.32
Camphor	0.76	0.6	0.26	0.47
4-terpineol	0.48	0.69	0.46	-
Borneol	-	-	-	0.44
α-terpineol	0.79	1.18	0.98	1.44
Endobornyl actate	0.97	1.45	1.63	1.46
Methyl cinnamate	1.6	1.5	1.78	0.67
β-caryophyllene	0.01	-	-	-
α-copaene	0.01	0.01	0.07	0.01
Eugenol	7.16	8.83	9.69	12.64
Methyl eugenol	13.83	8.91	10.7	5.68
α-bergamotene	-	-	1.78	-
α-cubebene	0.01	0.07	0.11	0.02
α-humulene	0.83	0.84	0.95	1.05
δ-muurolene	-	0.09	-	0.01
Germacrene D	0.96	0.4	2.64	0.78
Bicycloge- macrene	0.59	0.68	1.59	0.74
α-guaiene	0.23	0.43	-	0.5
δ-cadinene	1.66	2.27	2.22	2.55
α-muurolene	0.04	0.08	0.05	0.09
Nerollidol	0.13	0.12	1.19	0.13
Spathulenol	0.06	0.05	0.16	0.03
Iso-spathulenol	0.04	0.04	0.11	0.04
Carotol	0.5	0.61	1.18	0.71
α-cadinol	4.46	4.94	9.59	5.21
α-eudesmol	-	0.35	0.65	0.25
α-bisabolol	-	0.05	0.09	0.08
Farnesol	-	0.03	-	0.04

Table 2: RAPD and ISSR primer names and sequences

Type of marker	Primer name	Sequence (5.....3)
RAPD	OPA-08	GTG ACG TAG G
	OPA-10	GTG ATC GCA G
	OPA-11	CAA TCG CCG T
	OPA-15	TTC CGA ACC C
	OPC-B15	GGA GGG TGT T
ISSR	HB1	$(CT)^8TG$
	98B	$(CA)^3$ $(TA)^3$ GT
	HB08	$(GA)^6GG$
	HB09	$(GT)^6$ GG
	HB10	$(GA)^6$ CC
	HB12	$(CAG)^3GC$
	HB15	$(GTC)^3GC$
	844A	$(CT)^8GC$
	44B	$(CT)^8$ GC
	49B	$(CA)^3$ $(TA)^3$ GG

were analyzed using 0.8% agarose gel electrophoresis and visualized with 10 ìg/il ethidium bromide staining. The sizes of the fragments were estimated based on a DNA ladder of 100 bp.

SDS-PAGE Protein Analysis: The electrophoretic patterns of soluble proteins were detected by sodium dodecylsulfate polyacrylamide gel electrophoresis technique (SDS- PAGE). Then, 50µl from each liver extract was added to 25µl 2X lan's buffer, 10 µl mercaptoethanol (10% v/v) and drop of bromophenol blue in each tube, then all samples were boiled for 5 min and loaded into wells. The gels were run at 200V/15 min and then the volt was raised to 250 V/until the run ends (3-4hrs). All buffers solutions, stock solution, stock solution, staining and destaining solutions and gel preparation of protein were prepared according to Laemmli [22] as modified by Studier [23].

Statistical Analysis: The amplified bands were scored as 1 and 0 based on band presence and absence, respectively. Sizes of amplified bands were estimated using Gel Pro analyzer software. The binary data set was used to calculate the pairwise Jaccard similarity index and to assemble the corresponding similarity matrix. The matrix obtained was used to generate a dendrogram using the UPGMA method (Unweigthed Pair Group Method Arithmetical Means). All the analyses were performed using Non linear Dynamics -PC program.

RESULTS AND DISCUSSION

Essential Oil: Main components of the essential oil (Table 1 and Fig. 1) showed that the *Ocimum basilicum* essential oil appeared to be complex and riche in flavor notes and have higher proportions of linalool representing 46.63% in the oil, but intermediate proportions of methyl eugenol (13.83%), 1, 8-cineol (13.20%), eugenol (7.16%) and α-cadinol (4.46%). Nacar and Tansi [24] found that the major oil components of sweet basil was linalool, eugenol and germcrene D accompanied by lesser amount of α-pinene, camphene, sabinene, β-pinene, myrcene, 1, 8 -cineol, cis-ocimene, terpinolene, camphore, borneol, 4 -terpinol, α-terpineol, bornyl acetate, α-cubebene, α-copaene, β-elemene, methyleugenol, β-caryophyllene, α-humulene, α-amorphene, biclogermacrene, guaiene and cadinene. The results indicated that most percentage of the oil was oxygenated compounds with much higher quantities of linalool and 1, 8 -cineol (Table 1), but also

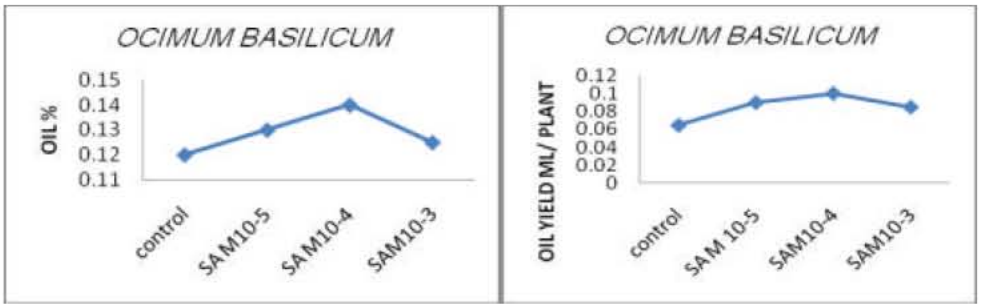

Fig. 1: Effect of foliar spray with salicylic acid on the oil content of *Ocimum basilicum* L

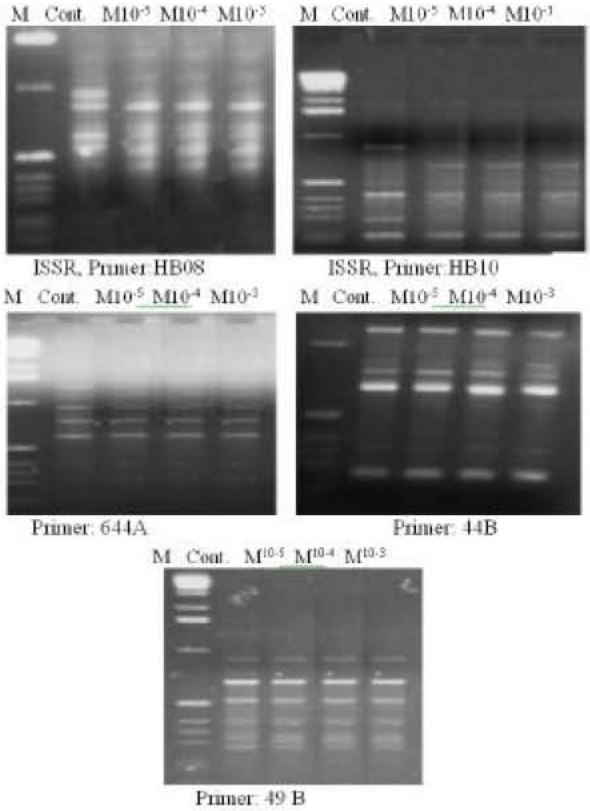

Fig. 2 : ISSR profiles of DNA genome of Ocimum basilicum control and other three which foliar spray with salicylic acid $M10^{-5}$, $M10^{-4}$, and $M10^{-3}$ concentration using primers: BH 08, HB 10, 644A, 44B and 49 B. The amplification products were separated on agarose gel and stain with ethidium bromide. Cont. = control plant, $M10^{-5}$= plant treated with foliar spray of Salicylic acid with concentration $M10^{-5}$, $M10^{-4}$, treated plant with $M10^{-4}$ of salicylic acid and $M10^{-3}$ treated plant with concentration $m10^{-3}$.

other compounds are nearly similar to our findings. The oil of Egypt basil can be classified as Linalool Rich Type [24]. However, there was a pronounced difference in the essential oil of Ocimum basilicum content due to SA application. Common components of essential oil in all treatments were linalool (46.63 - 43.32%), methyl eugenol (13.83 - 5.68%), 1, 8 -cineol (13.20 - 4.43%), eugenol (12.64 - 7.16%) and α-cadinol (9.59 - 4.46%). In addition, SA application increased the eugenol level from 7.13% in control to 12.64% at maximal yield with SA at 10– 3 M and was accompanied by an increase in 1, 8 –cineol content (from 13.20 to 15.31%), all other components exhibited a slight change. In this regard, the relative percentage of eugenol was correlated with the antioxidant activity of Sweet basil essential oil in two assays [25]. Therefore, a high percentage of eugenol in

Table 3: ISSR analysis of *Ocimum basilicum* foliar spray with salicylic acid using ten primers

Band Number	Molecular weight (bp)	RF Values	Primer: HB1 Control	$M10^{-3}$	$M10^{-4}$	$M10^{-3}$
Band 1	685	0.26	1	0	0	0
Band 2	630	0.31	1	1	1	1
Band 3	568	0.35	1	1	0	0
Band 4	448	0.45	1	1	1	1
Band 5	345	0.66	1	1	1	1
Band 6	229	0.83	1	1	1	1
Band 7	180	0.93	1	1	1	1
Band	MW (bp)	RF value	Primer: 98B			
1	210	0.13	1	1	1	1
2	289	0.23	1	1	1	1
3	293	0.29	1	0	0	0
4	304	0.35	1	1	1	1
5	318	0.45	0	1	1	1
6	323	0.49	1	1	1	1
7	331	0.55	1	1	1	1
8	343	0.63	0	1	1	1
Band	MW (bp)	RF value	Primer: HB08			
1	277	0.13	1	1	1	1
2	289	0.23	1	1	1	1
3	297	0.29	1	0	0	0
4	304	0.35	1	1	1	1
5	318	0.45	0	1	1	1
6	323	0.49	1	1	1	1
7	331	0.55	1	1	1	1
8	343	0.63	0	1	1	1
Band	MW (bp)	RF value	Primer: HB09			
Band 1	96	0.16	1	1	1	1
Band 2	120	0.2	1	1	1	1
Band 3	168	0.28	1	1	1	1
Band 4	216	0.36	1	1	1	1
Band 5	247	0.71	1	1	1	1
Band 6	325	0.54	1	1	1	1
Band 7	351	0.41	1	1	1	1
Band	MW (bp)	RF value	Primer: HB10			
1	740	0.49	1	0	0	0
2	505	0.6	1	1	1	1
3	383	0.68	1	1	1	1
4	311	0.74	1	1	1	1
5	290	0.76	1	1	1	1
6	212	0.85	1	0	0	0
7	155	0.94	1	1	1	1
Band	MW (bp)	RF value	Primer: HB12			
1	618	0.5	1	1	1	1
2	347	0.73	1	1	1	1
3	257	0.85	1	1	0	0
4	211	0.93	1	1	1	1
Band	MW (bp)	RF value	Primer: HB15			
Band 1	952	0.28	1	1	1	1
Band 2	669	0.43	1	1	1	1
Band 3	436.5	0.53	1	1	1	1
Band 4	247	0.62	1	1	1	1
Band 5	279	0.7	1	1	1	1
Band 6	229	0.76	1	1	1	1
Band 7	250	0.83	1	1	1	1
Band	MW (bp)	RF value	Primer: 844A			
1	787	0.38	1	0	0	0
2	691	0.42	1	0	0	0
3	607	0.46	1	1	1	1
4	453	0.55	1	1	1	1
5	350	0.63	1	1	1	1

Table 3: Continued

Band	MW (bp)	RF value	Primer: 44B			
1	740	0.06	1	1	1	1
3	514	0.24	1	1	1	1
4	474	0.28	1	1	1	1
5	379	0.39	1	1	1	1
6	234	0.63	0	1	0	0
7	191	0.73	1	1	1	1
8	150	0.85	1	1	1	1
Band	MW (bp)	RF value	Primer: 49B			
1	639	0.3	1	0	0	0
2	479	0.54	1	1	1	1
3	430	0.63	1	1	1	1
4	386	0.72	1	1	1	1
5	351	0.8	1	1	1	1
6	327	0.86	1	1	1	1

the SA treated herb is of particular interest in basil, which could constitute new sources of antioxidant phenolics in the diet [26] reported that SA applied on basil stimulated the growth and oil yield by enhancing photosynthesis and nutrient uptake. Basil may be a new source of antioxidant phenolics in the diet due to the greater production of eugenol by SA and All SA treatments enhanced total free amino acid, free proline, Put, Spd as well as total polyamines TPAs, whilst differently affecting the level of spermine. However, further investigations are required to elucidate the possible role of SA on plant growth regulating activity. Smetanska *et al.* [27] reported that SA induced the biosynthesis of aromatic and indole glucosinolates in turnip. Deschamps Cícero *et al.* [28] also studied regulation of essential oil accumulation in basil in response to elicitation and he found that treatment of basil plants with methyl salicylate resulted in a 71% increase of methylchavicol production at 72 h after initiation of treatment when plants were subjected to treatment for 48 h.

Inter Simple Sequence Repeat ISSR Analysis: The results of total amplified fragments of sweet basil (*Ocimum basilicum*) using ISSR analysis with 10 primers are presented in Table 2 and 3. The electrophoretic patterns of primers of ISSR in of four plants (three treated plant with foliar spray with salicylic acid $M10^{-3}$, $M10^{-4}$ and $M10^{-3}$ concentrations and control) are displayed with Table 3.All treated plants and control gave bands which is maximum number eight by using 89B, Hb10 and 44 B and reached to minimum number were four at BH12.

With the primer BH1, there were five monomorphic from the exhibited seven bands from amplification products of ISSR with molecular size ; 630, 448, 245,229 and 180 bps respectively). On other hand, band 1 with Molecular size 685 (pb) was specific band for control only rather other control while band 3 with molecular size 568 (bp) was absent with treated sample with $M10^{-4}$ and

M 10^{-3} (Table 3). Primer 98B showed that there were five monomorphic bands with molecular size; 210, 289, 304, 323 and 331 (bp). However, band 3 (Molecular size; 293(bp) was positive band with control only while band five and eight were negative band (M size, 318 (bp) and 343 (bp)with control.

In primer HB08, five monomorphic bands were recorded with molecular sizes; 277, 289, 304, 323 and 331 (bp). Bands 3 with molecular size 297 appear only with control and disappear with other treatment plants. While bands five and eight with molecular size 318 and 343 (bp) were negative with control and present with all other treatment plants (Table 3).Control and all treated plants exhibited seven monomorphic bands with primer HB09 and HB15 and while with primer HB10 had five monomorphic bands and two bands heteromorphy, 740 and 212 (bp) respectively. With the HB12 primer, there were four detected bands, three of which were monomorphic ones with molecular size 618, 347 and 211 (bp). However, band 3 with molecular size 257 bp did not appear with treated plants with M10^{-4} and M10^{-3}. There were six monomorphic bands with primer 44 B and only one heteromorphy with molecular size 234 (bp) appeared only with treated plant with M 10^{-3} of salicylic acid (Table 3).

The densitometer analysis by using primer 49B revealed that the total bands were 6 bands, 5 of them were monomorphic and only one band disappeared with all treated plants. (Table 3). El-Mokadem and Heikal [31] reported the retarding effect of cycocel (2- chloroethyl, trimethyl ammonium chloride) on the ornamental plants *Encelia furinosa* was significantly higher (by using morphological and molecular markers of RAPD and ISSR), this is support our result.

RAPD Analysis: RAPD analysis was performed to study the genetic variation of *Ocimum basilicum L* and other plants foliar sprayed with SA (10^{-5}, 10^{-4} and 10^{-3} M) using five primers as shown in Table 4 and Fig.3. Four primers yielded maximum amplification products with control and all treated plants. Primer OPA-10 generated uncommon band with DNA from plant treated with M10^{-4} at molecular size 1559 (bp) while plant treated with M 10^{-3} characterize by negative band at molecular size 2123 (bp). Several polymorphic bands were detected or had differences too small to provide information on the genetic variation. In OPA-11, a amplify bands were seven bands, six of them were monomorphic with control and all treated plants and one was polymorphic, it was at molecular size 2310 (bp), appeared with all treated plants only, disappeared with control.

Table 4: Densitometric analysis for RAPD –PCR of DNA genome of *Ocimum basilicum* foliar spray with salicylic acid using five primers

			Primer: OPA-08			
Band Number	Molecular Size (bp)	RF Values	Control	M10^{-3}	M10^{-4}	M10^{-3}
1	500.0	0.6	1	1	1	1
2	530.0	0.64	1	1	1	1
3	289.0	0.98	1	1	1	1
4	181.0	0.77	1	1	1	1
Band no.		RF.		Primer:	OPA-10	
1	2123	0.28	1	1	1	0
2	1559	0.31	0	0	1	0
3	1350	0.36	0	0	1	1
4	913	0.42	1	1	1	1
5	409	0.52	1	1	1	1
6	313	0.68	1	1	1	1
7	245	0.85	1	1	1	1
8	212	0.91	1	1	1	1
Band no.		RF.		Primer:	OPA-11	
1	768	0.34	1	1	1	1
2	683	0.42	1	1	1	1
3	620	0.48	1	1	1	1
4	520	0.51	0	1	1	1
5	469	0.57	1	1	1	1
6	381	0.74	1	1	1	1
7	357	0.84	1	1	1	1
Band no.		RF.			Primer:	
1	2310	0.51	0	1	0	0
2	1528	0.53	1	0	1	1
3	1387	0.58	1	1	1	1
4	1046	0.61	1	1	0	0
5	851	0.64	1	1	1	1
6	768	0.67	1	0	0	0
7	624	0.71	0	1	1	1
8	399	0.77	1	0	0	0
9	248	0.85	0	1	1	1
10	303	0.92	1	1	1	1
Band no.		RF	Primer:	OPC-B15		
1	2112	0.19	1	1	1	0
2	1559	0.24	0	0	1	1
3	1409	0.33	1	1	1	1
4	1362	0.44	1	1	1	1
5	1273	0.57	0	0	0	1
6	1199	0.61	1	1	1	1

Table 5: Densitometric analysis for protein of *Ocimum basilicum* foliar spray with salicylic acid (M 10^{-5}, M10^{-4} and M10^{-3})

Band Number	Total MW (KDs) Values	Control	M10^{-5}	M10^{-4}	M10^{-3}
1	96	+	+	+	+
2	87	+	+	+	-
3	85	+	+	+	+
4	78	+	+	+	-
5	73	-	-	+	-
6	72	+	+	-	-
7	66	+	+	+	+
8	59	+	+	+	+
9	52	+	+	+	+
10	47	+	+	+	+
11	43	+	+	-	+
12	41	+	+	+	+
13	35	+	+	+	+
14	32	+	+	+	+
15	29	+	+	+	+
16	27	+	+	+	+

(MW) Molecular weight (+) presence of band -) absence of band

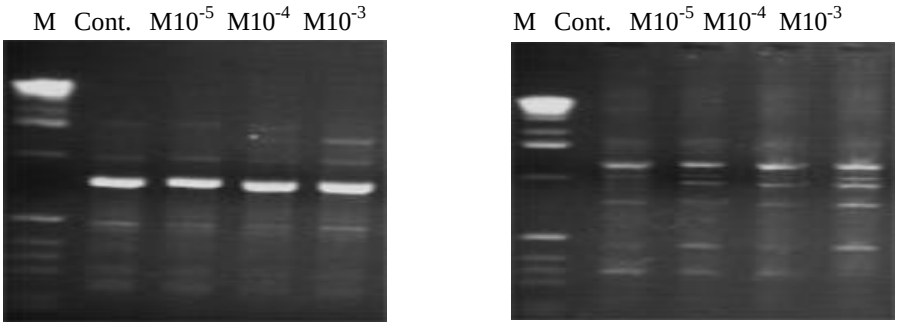

Fig. 3: RAPD profiles of DNA genome of *Ocimum basilicum* control and other three which foliar spray with salicylic acid $M10^{-5,}$ $M10^{-4,}$and $M10^{-3}$ concentration using primers:OPA-10 and OPA-11. The amplification products were separated on agarose gel and stain with ethidium bromide. Cont,= control plant, $M10^{-5}$= plant treated with foliar spray of Salicylic acid with concentration $M10^{-5,}$ $M10^{-4}$, treated plant with $M10^{-4}$ of salicylic acid and $M10^{-3}$ treated plant with concentration $m10^{-3}$.

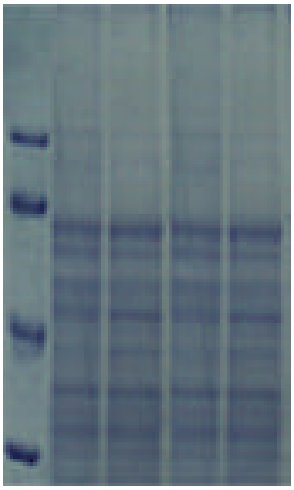

Fig. 4: SDS protein bands of *Ocimum basilicum* control and other three which foliar spray with salicylic acid $M10^{-5}$, $M10^{-4}$,and $M10^{-3}$ concentration

Phylogenetic Tree of ISSR Data:

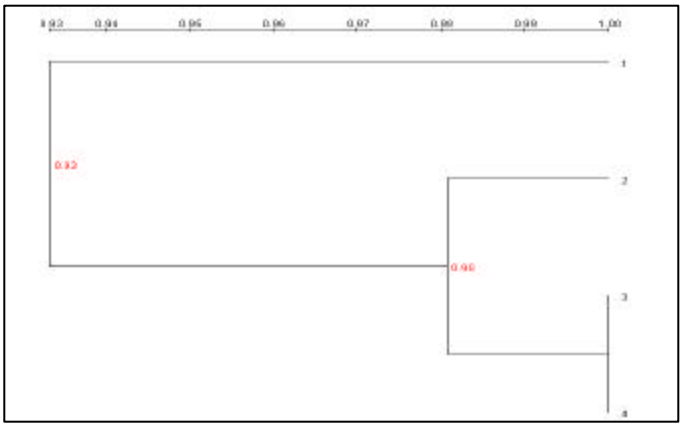

Fig. 5: Dendrogram for the Phylogenetic e relationships among *Ocimum basilicum* (control and other three which foliar spray with salicylic acid $M10^{-5}$, $M10^{-4}$,and $M10^{-3}$ concentration) based on similarity indices data of ISSR analysis

Phylogenetic Tree of RAPD Data:

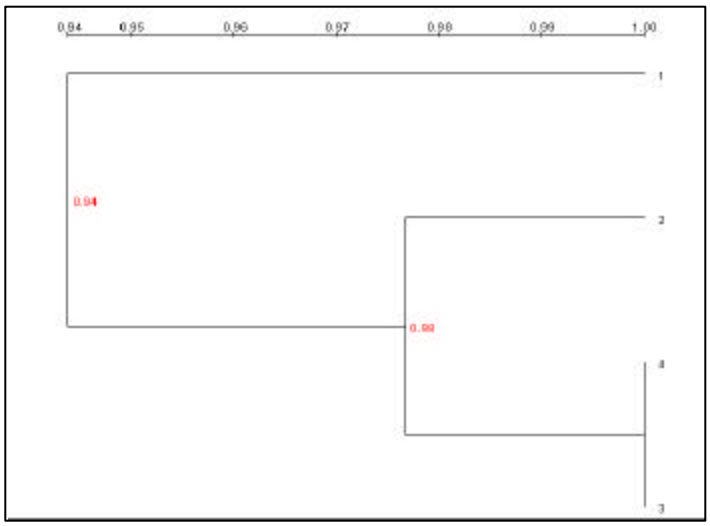

Fig. 6: Dendrogram for the Phylogenetic e relationships among *Ocimum basilicum (*control and other three which foliar spray with salicylic acid $M10^{-5}$, $M10^{-4}$,and $M10^{-3}$ concentration) based on similarity indices data of RAPD analysis

Phylogenetic Tree OF Combined Data:

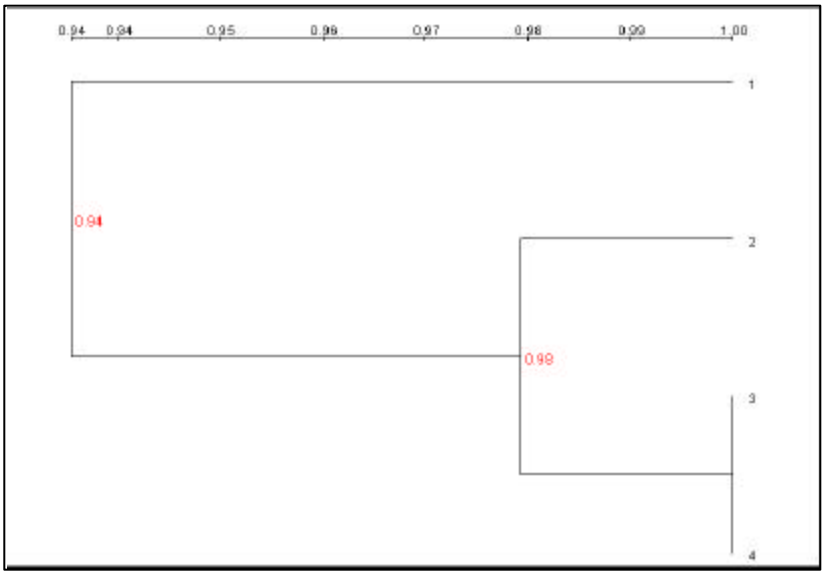

Fig. 7: Dendrogram for the Phylogenetic e relationships among *Ocimum basilicum* (control and other three which foliar spray with salicylic acid $M10^{-5}$, $M10^{-4}$,and $M10^{-3}$ concentration) based on similarity indices - combined data of RAPD and ISSR analyses

Primer OPC-b15 revealed three monomorphic bands were recorded with molecular size 1387, 851 and 303 (bp). Band one with molecular size 2310 (bp) appear only with treated plant with Salicylic acid ($M10^{-5}$) and disappeared with control and other treated plants. Band with molecular size (1046 bp) missed with treated plant with Salicylic acid($M10^{-3}$ and $M10^{-4}$compared with control and other treated plants. Control was characterized by two positive bands at molecular size 768 and 399 bps) and negative band at245 bp.Treated plant with salicylic acid ($M10^{-3}$) characterized by two bands; one negative with molecular size 2112 (bp) and other positive with molecular size 1273 (bp). Vieira *et al.* [29] studied genetic variation of *Ocimum gratissimum L.* based on volatile oil constituents, flavonoids and RAPD markers and found that the relationship between RAPDs and these chemical markers. Also Luigi De Masil *et al.* [30] support the result by using *Ocimum basilicum* L.

ISSR and RAPD techniques indicated that they are useful in the estimation of Phylogenetic relationships among the control and the treated samples with SA. In spite of differences in the number of used ISSR –primers (10) and RAPD primers (5), they showed nearly equal efficient in the detection of polymorphism (Fig. 5-7).

SDS- Protein Analysis: Water soluble proteins were analyzed using SDS-PAGE based on the variation in their molecular weights using SDS protein standard markers. The electrophoretic patterns of the SDS- water protein of treated plants and control exhibited a maximum number of exhibited a total of 16 bands. Table 5 and Fig. 4 showed that there were slightly differences between the treated plants and control. The treated plant with SA $M10^{-4}$ characterized by three specific bands; one positive band at 73 KDa and two negative bands at 72 and 43 KDa comparing with control and other treatments. Also treated plants with SA $m10^{-3}$ had two specific bands at 87 and 78 KDa respectively. Hoyos Mary Elizabeth and Zhang Shuqun [32] and Mikoajczyka Monika [33] reported that proteins play important roles in signaling the plant adaptive responses to salinity/drought stresses and salicylic acid-induced protein kinase with a member of the tobacco plant. Consequently the appeared bands of protein and disappeared of other due to the effect of salicylic acid on these plant.

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