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CHEMICAL COMPOSITION AND TOXIC EFFECTS OF ESSENTIAL OIL OF *Origanum rotundifolium* BOISS. ON *Zea mays* SEEDLINGS

Origanum rotundifolium BOISS.'İN UÇUCU YAĞLARININ KİMYASAL BİLEŞİMİ VE *Zea mays* TOHUMLARININ ÇİMLENMESİ ÜZERİNE TOKSİK ETKİLERİ

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Abstract

This study was conducted to investigate essential oil based genetical and physiological changes on maize seedling under laboratory conditions. The aerial parts of *O. rotundifolium* BOISS. were powdered, and EO obtained by hydro-distillation. For the best results, the content applied to Gas Chromatography/Mass Spectrometry (GC-MS), and the main constituents have been determined as *Carvacrol* (%43.62) and *Thymol* (%40.86). The liquid phase of the oil applied to maize seeds at four concentrations. Randomly Amplified Polymorphic DNA method was used to observe possible DNA alterations. With the total soluble protein level and SDS Page analysis, the physiological effects of EO were determined. The oils showed phytotoxic effects, but not in a dose-dependent manner. Soluble protein level and SDS Page analysis did not show any significant change but genomic template stability value was decreased with the increasing of EO dose.

Keywords: DNA change; Essential Oil; SDS PAGE; GC-MS; Soluble Protein Level; RAPD

Özet

Bu çalışma; uçucu yağların laboratuvar şartlarında mısır fidelerinde neden olduğu genetik ve fizyolojik hasarları belirlemek amacıyla yapıldı. *O. rotundifolium* BOISS. bitkisinin toprak üstü organları toz haline getirilip hidrodistilasyon yöntemiyle uçucu yağları elde edildi. Doğru sonuç elde etmek için uçucu yağların içeriği GC-MS yöntemiyle belirlenip temel bileşenleri belirlenip en fazla bulunan içeriklerin %43.62 oranıyla *Carvacrol* ve %40.86 oranıyla *Thymol* olduğu tespit edildi. Sıvı haldeki toplam içerik mısır tohumlarına dört farklı dozda uygulandı. RAPD analizleriyle olası genetiksel değişimler belirlendi. Toplam çözünür protein miktarı ve SDS Page profilleriyle ise fizyolojik değişimler tespit edildi. Uçucu yağların dozdan bağımsız fitotoksik etki yarattığı gözlendi. Genomik stabilitenin artan uçucu yağ konsantrasyonuna bağlı olarak düştüğü ancak bu değişimin çözünür protein miktarı ile protein profillerini etkilemediği belirlendi.

Anahtar Kelimeler: DNA değişimi; Esan siyel yağ; SDS PAGE; GC-MS; Çözünür protein miktarı; RAPD

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1. INTRODUCTION

The genera belong to Lamiaceae plant family is capable of to interact with nearest plants, and protect them against any insect attack. This interaction starts with the releasing of Essential Oil (EO), which are the complex mixture of volatile compounds produced by these plants. *Origanum* is the one of this genera, has about 26 aromatic species (including subspecies) in Turkey. *Origanum rotundifolium* is the one, which we have studied in this work. Mostly, the biological activities of EO of this species were studied as antibacterial [1], antioxidant [2], like other EO based studies. Besides, the scientists have focused on allelopathic potential of the mentioned oils or their derivates [3]. This feature of the oils makes them considerable, especially, on agricultural studies.

Simply, we can explain allelopathy as the effect of one plant on another plant, which can be positive or negative. These interactions are based primarily on the production of secondary chemicals by higher plants that produce a wide array of biochemical compounds that create biological changes [4]. With the determination of the chemical constituent of EO, studies turned on mode of action of the compounds. For example, some of the chemical content of EO, 1,8-cineole and 1,4-cineole are strong growth inhibitors, and 1,8-cineole strongly inhibits all stages of mitosis [5]. On the other hand, previous study indicates that, some monoterpenes, namely α-pinine, β-pinine, cineole, camphore, have suppressed the growth of herbaceous plants [6]. Furthermore, cytotoxic effect of some constituents was known. Phytotoxic potential of the monoterpenes proposed them to use as a potential biocontrol matter like herbicide [7]. With the application of the chemicals to the agricultural areas cause negative effects on both agriculture and human being [8, 9], so the using of this natural occurred compounds as “bioherbicide” idea become popular. The scientific community must be careful to avoid the previous mistakes. Therefore, EO based DNA damages must be clarified for further uses.

Depending on this information, we aimed to determine the genetic and physiological effects of EO on model plant maize in the current study. Randomly Amplified Polymorphic DNA (RAPD) technique, which is recently become popular on toxicological studies, was applied for genetical changes. On the other hand, for determining of the physiological effects of oils, total soluble protein levels of treated samples were estimated, and with the SDS-Page analysis we try to explain the effects of EO on protein profile. For the better understanding GC-MS analysis of the oil was performed too.

2. MATERIALS AND METHODS

2.1. Plant Material

Origanum rotundifolium were collected at the flowering stage in June 2009, in the northeast part of Erzurum city of Turkey. The identification of plant materials was con-

firmed by Assist. Prof. Dr. Ozkan AKSAKAL, in the Department of Biology, Ataturk University, Erzurum, Turkey.

The maize seeds were obtained from Department of Field Crops, Faculty of Agriculture, Ataturk University, Erzurum, Turkey.

2.2. Isolation of Essential Oils

Plant samples were seared in a canopy room. The leaves and flowers of the plant were powdered with blender and then subjected to water distillation for 2-3 h in a Clevenger-type apparatus (Thermal Laboratory Equipment, TURKEY). The obtained EO was stored at +4 ºC for further studies.

2.3. GC–MS Analysis

The analysis of the essential oils was performed using a Thermofinnigan Trace GC/ Trace DSQ/A1300, (E.I Quadrapole) equipped with a SGE-BPX5 MS capillary column (30 m X 0.25 mm i.d., 0.25 µm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was the carrier gas, at a flow rate of 1 mL/min. Injector and MS transfer line temperatures were set at 220 ºC and 290 ºC respectively. The program was used at 50-150 ºC at a rate of 3 ºC/min. Diluted samples (1/100,v/v, in methylene chloride) of 1.0 µL were injected manually and in the splitless mode. The components were identified based on the comparison of their relative retention time and mass spectra with those of standards', Wiley 7N library data of the GC-MS system and literature data. The results were also confirmed by the comparison of the compounds' elution order with their relative retention indices on non-polar phases reported in the literature.

2.4. Seed Germination and Application of Essential Oils

Same size maize seeds were chosen, and their surface was sterilized with 5% (w/v) NaOCl for 6-10 minutes. After sterilization, seeds were washed with double-distilled water for five times and dried with the sterile filter paper. 15cm diameter Petri dishes on two layers of sterile Whatman No. 1 filter paper were used for germination. The seeds were exposed to four different (0.1, 0.2, 0.4, 0.8µl/ml) concentrations of EO. Tween 20 was used as solvent. 10 ml of this mixture and convenient EO dose were mixed and added to petri dishes. Only double distilled water with Tween 20 was used for the control group. Three replicates were made for each concentration. The petri dishes were allowed to germinate at 23 ˚C in the incubator (Binder, Tuttlingen, Germany) for a week.

2.5. Genomic DNA Isolation and RAPD Procedures

After the seed germination, the sufficient amount of seedlings was collected and

ground in liquid nitrogen. Genomic DNA obtained according to Li and Quiros [10]. For determining the primers that were used in the study, control DNA was treated with 30 different 10-base primers supplied by Operon. Sixteen of these primers produced amplicons with control DNA (Table 1).

Table 1. Sequences of 16 primers used in the study

No	Primer	Sequences of primers	
1	**OPA-13**	5'→3'	CAGCACCCAC
2	**OPH-17**	5'→3'	CACTCTCCTC
3	**OPA-2**	5'→3'	TGCCGAGCTG
4	**OPA-1**	5'→3'	CAGGCCCTTC
5	**OPA-6**	5'→3'	GGTCCCTGAC
6	**OPH-14**	5'→3'	ACCAGGTTGG
7	**OPH-18**	5'→3'	GAATCGGCCA
8	**OPY-6**	5'→3'	AAGGCTCACC
9	**OPY-1**	5'→3'	GTGGCATCTC
10	**OPY-8**	5'→3'	AGGCAGAGCA
11	**OPY-15**	5'→3'	AGTCGCCCTT
12	**OPY-16**	5'→3'	GGGCCAATGT
13	**OPW-1**	5'→3'	CTCAGTGTCC
14	**OPB-8**	5'→3'	GTCCACACGG
15	**OPW-7**	5'→3'	CTGGACGTCA
16	**OPW-5**	5'→3'	GGCGGATAAG

PCR conditions arranged as before stated in Agar et al., [11]. For electrophoresis, 10 µl of the PCR product mixed with 6x gel loading buffer (3 µl) and loaded onto an agarose (1.2% w/v) gel included EtBr solution (2 µl Etbr/100ml 1xTBE buffer) in 0.5xTBE (Tris-Borate- EDTA) buffer and was subjected to electrophoresis at 80 V for 150 min. The amplified DNA products were detected by using the Bio Doc Image Analysis System with Uvisoft analysis package (Cambrige, UK). The gels were estimated with Total Lab. TL 120 v.2009. The following formula was used for calculation of genomic template stability:

$$GTS = (1 - \frac{a}{n})x100$$

The average number of polymorphic bands detected in each treated sample (*a*) and the

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5	**OPA-6**	5'→3'	GGTCCCTGAC
6	**OPH-14**	5'→3'	ACCAGGTTGG
7	**OPH-18**	5'→3'	GAATCGGCCA
8	**OPY-6**	5'→3'	AAGGCTCACC
9	**OPY-1**	5'→3'	GTGGCATCTC
10	**OPY-8**	5'→3'	AGGCAGAGCA
11	**OPY-15**	5'→3'	AGTCGCCCTT
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$$GTS = (1 - \frac{a}{n})x100$$

The average number of polymorphic bands detected in each treated sample (*a*) and the

number of total bands in the control (*n*) were used for calculation.

2.6. Quantitative Analysis of Proteins and SDS-Page Procedure

Total soluble protein was measured as described by Bradford [12]. 0.5 g of each root and stem of EO treated and untreated samples were grinded and homogenized in 0.05M Phosphate buffer (pH: 6.5). Then the homogenates were centrifuged at 15000 rpm for 20 minutes. Protein concentration was estimated according to Bradford [12] using bovine serum albumin as the standard. Inhibitory rate (%) was calculated using the equation:

$$(1-\frac{x}{y})100$$

Where y and x are the average values detected in the control, and each sample treated respectively.

The procedure described by Shultz et. al., [13] was applied to samples with modifications for SDS-Page analyses. 0.1g of plant samples were powdered in liquid nitrogen and 2 ml of 0.05M Phosphate buffer (pH: 6.5) was added and mixed. Then the samples were centrifuged at 12000rpm for 10 minutes. Then 1 mL of TCA-acetone (90% acetone, 10% TCA, 0.07% BME) was added to the supernatant. 1g crystal TCA solved in 0.7 ml distilled water was used. Samples were vortexed, incubated at -20 °C for 30-45min, and centrifuged at 6,000 rpm for 6 min. Protein pellets were washed twice with acetone/BME and dried until all of acetone removed. Dried samples were re-suspended with SDS loading buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol and bromophenol blue as tracking dye), denaturized at 95 °C for 5 min and loaded on to gel. SDS-Page was carried out using 12% acrylamide gels [14]. The duration time of the electrophoretic separation was about 90 min. After the separation was finished, the gels were placed in a staining solution (0.1% w/v Coomassie Brilliant Blue R-250) for 45 min and then in solution I (=removing dye; methanol 50%, distilled water 40% and acetic acid10%) for 30 min. After this step, the gels were placed in solution II (=wash Solution; acetic acid7%, Methanol5% and distilled water88%) and examined.

2.7. Statistical Analysis

The statistical analyses were carried out using the package software SPSS 17.0 for windows. The change of the total soluble protein level was tested statistically by using One way ANOVA variance analyses.

3. RESULTS

3.1. Essential Oil Content of *Origanum rotundifolium*

The EO composition of *Origanum rotundifolium* listed in Table 2. The table shows

the retention indices (RIs) and the percentages of the detected compounds. Ten components that make up 100% of the oil composition were identified. The major components are determined as Carvacrol (43.62%) and Thymol (40.86%). The minor component was determined as Aromadendrene at 0.22% ratio.

Table 2 . Essential oil content of *Origanum rotundifolium*

	*RI	**RT	Components	(%)	Identificitation methods
1	1034	14.21	p-Cymene	5.95	GC, MS, RI
2	1067	15.74	γ–Terpinene	1.52	GC, MS, RI
3	1079	16.61	cis-Sabinenehydrate	0.56	GC, MS, RI
4	1172	21.58	Borneol	2.49	GC, MS, RI
5	1178	21.99	Terpinen-4-ol	0.63	GC, MS, RI
6	1289	27.34	Thymol	40.86	GC, MS, RI
7	1296	27.72	Carvacrol	43.62	GC, MS, RI
8	1419	32.23	β-Caryophyllene	2.44	GC, MS, RI
9	1442	33.05	Aromadendrene	0.22	GC, MS, RI
10	1579	39.58	Caryophyllene oxide	1.71	GC, MS, RI

*RI: Retention Index, **RT:Retention Time

3.2. The DNA Profile of Maize Treated with EO According to RAPD Procedure

Totally, 15 primers produced reproducible and polymorphic bands. According to RAPD profile totally 120 bands amplified by these primers with control DNA. The molecular size of amplified bands ranged from 47 (OPA 8) to 1515 (OPH 17) in control seedlings. Table 3 represents the DNA alterations, including loss of normal band and appearance of new bands, compared with the control seedlings. The samples treated with 0.1 µl/ml dose of EO displayed higher variation in the number of bands in RAPD analysis. It was observed that after EO treatment, the number of new bands was 14 and the numbers of disappeared bands were 46. The polymorphism value was calculated 36.66%, 25, 10.83, and 21.66 for 0.1, 0.2, 0.4 and 0.8 µl/ml doses, respectively (Table 3). On the other hand, Figure 1 presents a RAPD profile of OPW-1, OPB-8, OPW-7 and OPW-5 respectively. Based on these alterations GTS values was changed and calculated as 63.34%, 75%, 89.17%, 78.34% for 0.1, 0.2, 0.4 and 0.8 µl/ml doses, respectively (Table 3, Figure 1).

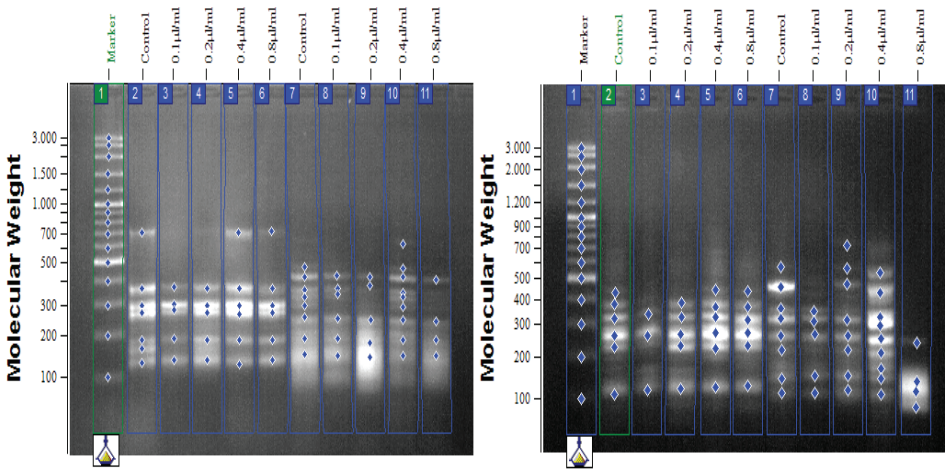

Figure 1. RAPD profiles of essential oils treated and non-treated (control) *Zea mays* seedlings with four different primers

Table 3. The number of bands in control and molecular size of (base pair) disappearance (-), and/or appearance (+) of DNA bands for all primers in the EO treated maize seedlings.

Primer	*Origanum rotundifolium*							
	Control	0.1µl/ml		0.2µl/ml	0.4µl/ml		0.8µl/ml	
OPA-13	7	+ -	*ND 358	396 ND	ND ND		ND 470	
OPH-17	7	+ -	ND 1515,495,372	ND 1515,495,372,265	554 ND		554 1515	
OPA-2	9	+ -	ND 461,405,341,261,128,94	ND 461,405	ND ND		ND 461,405	
OPA-1	9	+ -	ND 150	ND ND	ND ND		ND ND	
OPA-6	7	+ -	ND 658,350,239	715,531 658	715 239		715 239	
OPH-14	9	+ -	ND 936,831,713,642,520	556 936,831,642	ND 642		ND 936,831,642	

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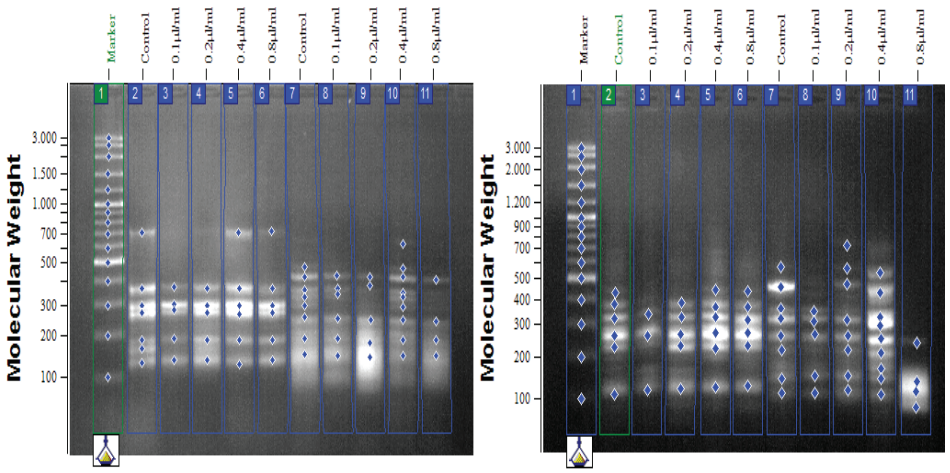

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OPH-14	9	+ -	ND 936,831,713,642,520	556 936,831,642	ND 642		ND 936,831,642	

OPH-18	4	+	416	416	934,416	ND
		-	ND	ND	ND	ND
OPY-6	10	+	ND	1027	1027	ND
		-	521,450,370,180,146	ND	ND	ND
OPY-1	6	+	ND	641,497	497	497
		-	565,345	ND	ND	ND
OPY-8	8	+	ND	ND	ND	ND
		-	830,649	830,649	649	649
OPY-15	8	+	ND	ND	ND	ND
		-	724,632	724,632	632	724,632
OPY-16	7	+	572	ND	ND	ND
		-	542,369	369	ND	542
OPW-1	7	+	ND	NDN	ND	ND
		-	705,165	705,165	165	165
OPB-8	8	+	ND	ND	628	ND
		-	469,300	469,332,300	ND	469,359,332,300
OPW-7	6	+	ND	ND	ND	ND
		-	517,486,324	517	ND	ND
OPW-5	8	+	ND	728	349	179
		-	582,528,316	ND	ND	582,528,466,424,316
Total Band	120		44	30	13	26
Polymorphism			36,66	25	10,83	21,66
GTS Value			63,34	75	89,17	78,34

*ND: None Detection

3.3. Effect of EO on Total Soluble Protein Level and SDS-Page Profile

Table 4 shows the soluble protein level of EO treated and untreated root and stem of maize seedlings, separately. Soluble protein content was increased in root samples, but not dose dependent. According to control, the soluble protein level was increased at 0.4 and 0.8µl/ml doses of stem. On the contrary, a decreasing was observed at 0.1 and 0.2µl/ml doses of them. The closest groups to the control were observed as 53.86 ug/ul (0.2 µl/ml) in the root and 53.21 ug/ul (0.2 µl/ml) in stem samples. Total soluble protein levels in seedlings varied noticeably ($P \leq 0.05$) compared with the control plantlets (Table 4).

Table 4. Total soluble protein of Root and Stem of *Zea mays* seedlings

ID	Conc [ug/ul]			
	Root	**IR***	**Stem**	**IR***
Control	52.20[a]	0	54.00[c]	0
0.1µl/ml	54.20[b]	3.83	52.34[a]	3.07
0.2µl/ml	53.86[b]	3.18	53.21[b]	1.46
0.4µl/ml	55.88[c]	7.04	55.19[d]	2.20
0.8µl/ml	54.52[b]	4,44	55.32[d]	2.44

Means within each column followed by the same letter are not significantly different at the P<0.05 level as determined by Duncan's multiple range test

*IR: Inhibitory rate %

According to SDS-Page analysis, the separated proteins showed bands ranged from20 to 86 kDa. The accumulation of the proteins and the intensity of the bands have shown variability. However, there are no significant changes on protein profile. Especially stem samples have not any change when comparing with control. Although, the protein accumulation was observed at root samples, the same effect has not seen on stem samples (Figure 2).

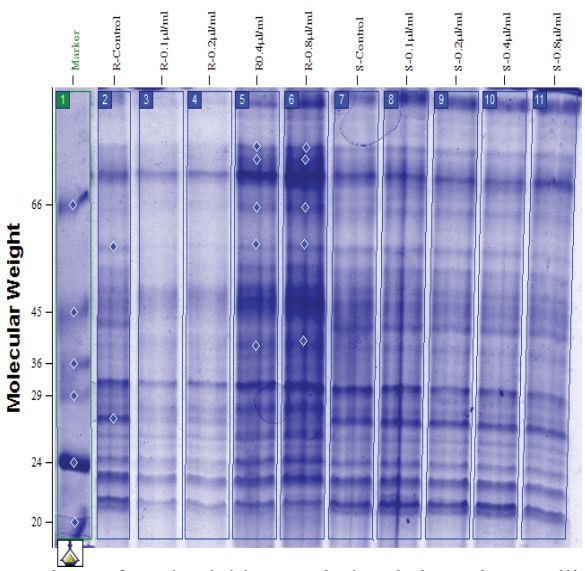

Figure 2. Comparison of total soluble protein levels in maize seedlings exposed to different concentrations of essential oils.

4. DISCUSSION

Plants have historically been the most important source of novel bioactive natural compounds. An important piece of these compounds is EO, which are mixtures of volatile compounds [15]. The uses of these oils have been varying. They have been applied against tumors for their potential anticancer effects, and also antinociceptive, antiviral, antiphlogistic, antioxidative [15], antimicrobial [16] potential of these compounds was studied. The diverse ecological functions of these compounds have been studied. They can act as internal messengers, as defensive substances against herbivores or as volatiles directing not only natural enemies to these herbivores but also attracting pollinating insects to their host [15]. Biologic activity of these compounds not limited with above-mentioned effects. Previous studies showed that these substances have allelopathic effects too. For example, Basile et al. [17] reported that EO of *Sideritis italic* caused a decrease in the percentage of seed germination and epicotyl growth in *Raphanus sativus* and a strong inhibition germination in two mosses. Furthermore, it was reported that, EO of *Shinus molle* had shown a dose dependent allelopathic activity on wheat germination and radical elongation [18]. The actual mode of action of EO against to these organisms is still unclear. In the current study, we aimed to be a part of the permanent solution of this problem. Because of the widely using of these substances, we need to clarify the mechanism of mode of action. There are just four studies about *O. rotundifolium.* The workers have focused on antioxidant [2], antibacterial [19, 20] and antimicrobial [21] studies. We do not come across any study about allelopathic or phytotoxic effects of this species. So we evaluate the allelopathic effects of EO of *O. rotundifolium* and its genetical mechanism. The current studiy was carried out in the laboratory. After the information about the mechanism of action -especially genetic one, the studies can turn on agricultural or other areas.

RAPD technique was used to determine toxicological or genetical changes on toxicant treated samples from the past to present [22, 23]. It is known that the technique is not costly even it is implemented quickly. For this reason, we applied RAPD technique to determine DNA changes.

After the application of EO to the maize seeds, they were recovered to designate inhibition of the germination. It was observed a certainly allelopathic effect but, not in a dose- dependent manner. In all EO treated doses, some of the seeds did not germinate. The germination ratio was decreased with the increasing of EO quantity (data not shown) but some of the seeds were germinated like control at the highest dose. It is well known that almost any substance that is inhibitory to a plant function at a particular concentration will likely prove stimulatory at some lesser concentration[24], so we can talk about synergistic or antagonistic effects of allelochemicals. It should be born of these possible effects of allelopathy. Accordingly, RAPD results have supported our findings (Figure 1). The changes occurred in GTS value may arise from the possible synergic effects of EO content. Previous study have same results with our findings [25]. Hong et al. [26] indi-

cated that, using thymol and ASM together against to some bacteria reduced some plant diseases than using thymol alone. In another study, carvacrol and eugenol had shown better inhibitory than alone uses of any of them [27]. The undulation of GTS values may be a result of synergistic effects of any these contents (Table 3). The same results were seen at the soluble protein level (Table 4). According to Table 4, protein level was changed, but irregularly. A decreasing was observed in stem protein level (0.1 and 0.2µl/ml), adversely an increasing was seen in root samples. The seeds can be subjected to an EO caused stress.

It is important that to determine the expression of the alterations occurred in RAPD profile (Figure 1). These alterations, including mutations and rearrangements not only change the genome profile, but also the protein profile too. According to SDS Page profiles, there is not a remarkable change except some protein accumulation (Figure 2). Especially, there is not any change on stem's protein profile. So RAPD changes can be ignored. The possible mechanism of allelopathy must be related genotoxic effects of oils.

5. CONCLUSION

As it has seen in discussion, the phytotoxic effects of natural occurred compounds may arise from discussed mechanisms. The alterations of DNA may occur in the inactive region of genome. However, SDS page profiles of proteins supported this theory. On the other hand, irregular variation on the total soluble protein level may be a result of plant stress. The germination stimulated hormones can cause seedlings too. The reason of the irregularity at both RAPD and protein profile should be a result of synergic or antagonistic effect of oils. The EO content must be decomposing, and should be apply separately for further studies.

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