MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES

INVESTIGATION OF GENETIC DIVERSITY, PHYLOGENETIC AND CHEMOTAXONOMIC RELATIONSHIPS IN SOME OPIUM POPPY (*Papaver somniferum* L.) CULTIVARS IN TURKEY

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Ph.D. THESIS
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Ugur SEN, a Doctor of Philosophy student of Marmara University Institute for Graduate Studies in Pure and Applied Sciences, defended his thesis entitled "**Investigation of Genetic Diversity, Phylogenetic and Chemotaxonomic Relationships in Some Opium Poppy (*Papaver somniferum* L.) Cultivars in Turkey**", on July 20, 2017 and has been found to be satisfactory by the jury members.

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APPROVAL

Marmara University Institute for Graduate Studies in Pure and Applied Sciences Executive Committee approves that Ugur SEN be granted the degree of Doctor of Philosophy in Department of Biology, Biology Program on 07.08.2017 (Resolution no:2017/18-95

Director of the Institute

Prof. Dr. Ugur YAHSI
Müdür

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The conference paper below has been accepted from this study;

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ABSTRACT

Investigation of Genetic Diversity, Phylogenetic and Chemotaxonomic Relationships in Some Opium Poppy (*Papaver somniferum* L.) Cultivars in Turkey

Papaveraceae family includes 28 genera and approximately 250 species around the world. Members of this family are spread on subtropical regions in Northern Hemisphere. In Turkey, there are five genus belonging to Papaveraceae family one of which is *Papaver* L. Within this genus members, *Papaver somniferum* L. (Opium poppy) is an annual and usually glaucous herb, and is known as "haşhaş" in Turkey. It is one of the sources of various pharmaceutically and economically beneficial alkaloids, fatty acids such as morphine, papaverine, codeine, noscapine and linoleic, oleic and palmitic acids.

In this study, chemical compounds were analyzed by using GC-FID and ICP-MS. As a result of our measurements, linoleic acid, oleic acid and palmitic acid showed the highest ratio in all studied cultivars, respectively. Hierarchical cluster dendogram was constructed according to the chemical compound analysis, and TMO T and TMO 3 cultivars were determined as the most distant relatives.

Some candidate genes/regions in plants have been proposed as possible standard markers, but none of them were commonly accepted by taxonomic communities. However, nuclear ribosomal DNA (nrDNA) ITS regions have been often used in phylogenetic studies. Additionally, chloroplastic *trnL-trnF* intergenic spacer is also employed to find out phylogenetic relationships between various plants. Also, inter simple sequence repeats (ISSR) markers are the most used markers for giving reproducible, highly polymorphic and informative results.

In this study, 18 ISSR primers were used in 12 different *P. somniferum* cultivars, which were widely used in Turkey and genetic diversity and phylogenetic relationship between these cultivars were investigated by using internal transcribed spacer (ITS) and *trnL-trnF* intergenic spacer. According to our results, two main groups were observed, and the most homogenous was Group A including TMO 2, Bolvadin 95, TMO T, Afyon 95, Ofis 96,

Ofis 3, Ofis 4 and Ofis 95 cultivars, while Group B including TMO 3, Ofis 8, TMO 4 and TMO 1 cultivars.

Keywords: Opium poppy, phylogeny, GC-FID, ICP-MS, ISSR, ITS, *trnL- trnF*

July, 2017 **Ugur SEN**

ÖZET

Türkiye'deki Bazı Haşhaş (*Papaver somniferum* L.) Kültür Varyetelerinde Genetik Çeşitlilik, Kemotaksonomik ve Filogenetik İlişkilerin Araştırılması

Papaveraceae familyası dünya üzerinde 28 cins ve yaklaşık 250 tür içermektedir. Bu familyanın üyeleri, kuzey yarımkürede subtropikal bölgelerde yayılım göstermektedir. Turkey'de ise 5 farklı cinsi bulunmaktadır ki *Papaver* L.'de bunlardan biridir. Bu cinse bağlı *Papaver somniferum* L. (haşhaş) tek yıllık ve genellikle pürüzsüz bitkidir ve Türkiye'de "haşhaş" olarak bilinmektedir. Farmasötik ve ekonomik açıdan değerli morfin, kodein ve noskapin gibi önemli alkaloidlerin, linoleik asit, oleik asit and palmitik asit gibi önemli yağ asitlerinin kaynağıdır. Bitkilerdeki pek çok gen/bölgeler olası bir standart işaretleyici olarak önerilmiştir fakat hiçbiri taksonomik topluluklar tarafından genel bir kabul görmemiştir. Bununla birlikte, nükleer ribosomal DNA (nrDNA) ITS bölgeleri, genellikle filogenetik çalışmalarda kullanılmıştır. Ek olarak bitkiler ve diğer organizmalar arasındaki araştırmalarda kloroplastik *trnL-trnF* spacer da kullanılmıştır. Ayrıca kısa dizi tekrarları arası (ISSR) işaretleyicileri tekrarlanabilir, oldukça polimorfik ve bilgilendirici sonuçlar vermek için en çok kullanılan işaretleyicilerdir.

Bu çalışmada kimyasal bileşen analizlerinin sonucunda hiyerarşik küme dendogramı oluşturulmuştur. TMO T ve TMO 3'ün en uzak akrabalık ilişkisine sahip olduğu belirlenmiştir. Türkiye'de yetiştirilen 12 adet haşhaş kültür varyetesi materyal olarak kullanılmıştır. Çalışma, 18 farklı ISSR tekniğine ait işaretleyici kullanılarak bitki genomlarındaki genetik çeşitlilik araştırılmış, ITS ve *trnL–trnF* intergenik spacer kullanılarak çeşitler arasindaki genetik ilişki ortaya konmuştur. Sonuçlara göre, iki ana grup gözlenmiştir. En homojen grubun , TMO 2, Bolvadin 95, TMO T, Afyon 95, Ofis 96, Ofis 3, Ofis 4 ve Ofis 95 varyeteleri içeren A grubu olduğu diğer grrup olan B grubu ise TMO 3, Ofis 8, TMO 4 ve TMO 1 varyetelerinden oluştuğu görülmüştür.

Anahtar kelimeler: Haşhaş, filogeni, GC-FID, ICP-MS, ISSR, ITS, *trnL- trnF*

Temmuz, 2017 **Uğur ŞEN**

CLAIM FOR ORIGINALTY

In this study, seed fatty acids, total proteins, heavy metals and mineral nutrients of 12 different Turkish opium poppy (*Papaver somniferum* L.) cultivars, which were recently registered by Turkish Grain Board were analyzed. For this aim, GC-FID and ICP-MS tools were used. Although there were similar studies realized by using these species, most of our cultivars have never been studied before. Additionally, a hierarchial chemotaxonomical analysis was realized by using SPSS statistical program with the obtained data.

Also, in another step of this study, genomic DNA was isolated and sequence analysis was done with ITS and *trnL- trnF* markers. Obtained genomic sequences were registered to National Center of Biotechnology Information (NCBI) by our study group. Then, phylogenetic relationships and genetic diversity of these varieties were analyzed using ITS and *trnL-trnF* regions and ISSR molecular markers, respectively. Both chemotaxonomic and genetic data were compared to determine whether there was any relationship between genetic and chemical properties of studied cultivars.

Therefore, this study includes new genetic and chemical data about 12 different recently registered Turkish opium poppy cultivars. Also, this study comprises our original works and analysis, and any materials have not previously published. We further declare that, this study does not contain any materials, which were submitted for any degrees, diplomas and qualifications at other institutions.

SYMBOLS

%	: Percentage
µl	: Microliter
^{o}C	: Celsius
rpm	: Revolutions per minute
M	: Molarity
cm	: Millimeter
g/cm^{3}	: Gram per cubic centimeter
ml	: milliliter
mm	: millimeter
V	: Voltage
v	: Volume
w	: Weight
µM	: Micromolar
µV	: Microvolt

ABBREVIATIONS

AFLP : Amplified Fragment Length Polymorphism
Ag : Silver
Al : Aluminum
As : Arsenic
ATP : Adenosine Tri-Phosphate
B : Boron
Ba : Barium
Ca : Calcium
Cd : Cadmium
Cr : Chromium
Co : Cobalt
Cu : Copper
DNA : Deoxyribonucleic acid
Fe : Iron
Hg : Mercury
ISSR : Inter simple sequence repeat
ITS : Internal transcribed spacer
K : Potassium
Li : Lithium
Mg : Magnesium
ML : Maximum Likelihood
Mn : Manganese
Mo : Molybdenum
Na : Sodium
NCBI : National Center of Biotechnology Information
Ni : Nickel
P : Phosphorus
Pb : Lead
PCR : Polymerase chain reaction
PEP : Phosphoenolpyruvate
RAPD : Random amplified polymorphic DNA
RNA : Ribonucleic acid
ROS : Reactive oxygen species
Sb : Antimony
Se : Selenium
Sn : Tin
Sr : Strontium
Ti : Titanium
Tl : Thallium
TPA : Total protein analysis
U : Uranium
V : Vanadium
Zn : Zinc
TPA : Total protein analysis

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1. INTRODUCTION

1.1 Description of Opium Poppy (*Papaver somniferum* L.)

1.1.1 Botanical Properties

Papaveraceae family, which belongs to Ranunculales order, includes 28 genera and approximately 250 species all around the world. This species spread on subtropical region in Northern Hemisphere. In Turkey, there are 5 genera that belong to Papaveraceae one of which is *Papaver* L. (Seçmen vd. 1995). *Papaver* genus has 36 recorded species in Turkey about 110 around the world. *Papaver* has annual, biennial and perennial herbs. Flowers are solitary, in racemes or pannicles and often showy. Fruit is a capsule that has openings by pore-like valves just beneath the persistent stigmatic disc (Davis, 1965). *Papaver* genus, 170 alkaloids of which are isolated, is grouped under 9 section (Tavakkoli and Assadi, 2016).

Table 1.1 The classification of *Papaver somniferum* L.

Rank	Taxonomic name
Kingdom	Plantae
Subkingdom	Tracheobionta - Vascular plants
Division	Magnoliophyta - Flowering plants
Subdivision	Spermatophyta - Seed plants
Class	Magnoliopsida - Dicotyledons
Order	Ranunculales
Family	Papaveraceae – Dicotyledons
Genus	*Papaver* - Poppy
Section	*Papaver*
Species	*Papaver somniferum* L.- opium poppy

Opium poppy (*Papaver somniferum* L.), which is a member of *Papaver* section (Hosokawa et al., 2003), is an annual and usually glaucous herb. Petals are orbicular, white, lilac, purple or variegated colour (Davis, 1965). *P. somniferum* is also known as "haşhaş" in Turkey which is a major opium poppy producer with the Irano-Anatolian region widely confirmed as the center of origin of this yield (Celik et. al. 2016), and

considered as an important source of beneficial alkaloids such as morphine, codeine, papaverine and noscapine (Facchini and De Luca, 2008). Owing to an abundance of its alkaloid content, opium poppy is considered as an influential pharmaceutical crop for many countries, where it's widely cultivated, such as Turkey, and India. Opium poppy is cultivated for many benzylisoquinoline alkaloids (BIAs) namely papaverine, codeine, noscapine and morphine used as antispasmodic, antitussive, anticancer and analgesic agents respectively (Alagoz et. al. 2016). In spite of having 50% of opium poppy croplands (Turkish Grain Board, 2009), Turkey is the third biggest morphine producer (18%). Due to lack of research on improving biochemical content (Celik et al. 2016), opium poppy cultivars in Turkey have low morphine content which leads to low crop effectivity.

As well as alkaloid contents, opium poppy seeds have rich fatty acid content which also makes them very valuable. From nutritional aspect, poppy oil has the desired ideal fatty acid composition. It mainly contains 73% linoleic, 10% palmitic and 13% oleic acid. It is known that linoleic acid rich vegetable oils such as corn and safflower oils reduce the serum cholesterol levels in blood, this is also an indicator for nutrition value of poppy oil.

400 tons of opium poppy worth $320 million is required for medicinal use of the world (Turkish Grain Board, 2014), however, number of molecular studies conducted with accurate marker systems is unsatisfying (Parmaksiz and Ozcan 2011). Consequently, some researches on expressed sequence tag (EST) and simple sequence repeat (SSR) markers have been made and it is proven that these markers can be utilized to investigate genetic diversity of opium poppy species (Selale et al. 2013). Despite of insufficient molecular research on Turkish germplasm, there are some studies on morphological variation of both local poppy species and its cultivars (Gumuscu et al., 2008). Arslan et al. (2000) investigated morphine contents of 353 opium poppy varieties. 20 opium poppy cultivars were also determined for morphine contents by Gumuscu and Arslan (1999). Gumuscu et al. (2008) studied on alkaloid contents of 99 Turkish opium poppy lines. It is obvious that there are lack of research on both morphological and molecular analysis of opium poppy in Turkey.

1.1.2 History and cultivation of opium poppy

The opium poppy has been planted for use as drug and food (cakes, cookies, bread etc.) since settled life of humanity (Huang and Kutchan, 2000). Opium poppy originated in Asia. The first and most ancient evidence relating the popy is given in a tablet of clay found at the spiritual center of the Sumerians (Nippur). Nippur is an ancient city at southeast of Iraq, University of Pennsylvania Museum of Archeology and Anthropology have undertaken small tablets (Krikitos and Papadoki, 1967). The Sumerians planted the poppy for opium aproximately B.C. 3000 and called it "Gil", which means happiness in Sumerian language. Assyrians had a name too for poppy plant; "arat-pa-pal". They had been called opium poppy as "Joy Plant". The Persians learned the opium poppy during their conquests of Babylon and Assyria from the local people in 600 B.C. Persians called opium "theriac", "malideh" or "afiun" (Kapoor, 1995).

In the Ebers papyrus (B.C.1553 or 1550) a medical text about poppy capsules were mentioned with other remedies. That was written "To stop a crying child: Pods of poppy plant, fly dirt which is on the wall, make it into one, strain and take for four days, it acts at once."(Bryan, 1930).

In Greek, the first written record about poppy was found in Hesiod's Theogony (Hesiodos-B.C. 750-650). The Ancient Greek poet portrayed the deities Thanatos (death), Hypnos (sleep) and Nyx (night) crowned with poppies or carrying poppies in their hands (Tekiner and Kosar, 2016).

Opium poppy had been cultivated in Arab Emirates during the seventh century A.D. Avicenna or Abu Ali Ibn Sina (A.D. 980-1037), the most famous Arabian physician and philosopher, wrote a thesis about opium. Historians say that Ibn Sina died by opium poisoning in Persia in 1037 A.D. Arabs called opium poppy as "Abou el-noum", which means "father of sleep". Arabs brought the opium to China (Kapoor, 1995).

The presence of opium poppy in Anatolia dates back to the Hittite Empire (B.C. 1400-1200). Two coins minted in Anatolia, one of which was made by Romans in 330 BC in Şuhut with a poppy fruit on it, and the other found in Emirdağ with figure of a goddess

holding a poppy bouquet in her hand supports the presence of opium poppy in Anatolia from ancient times (Akcicek, 1997).

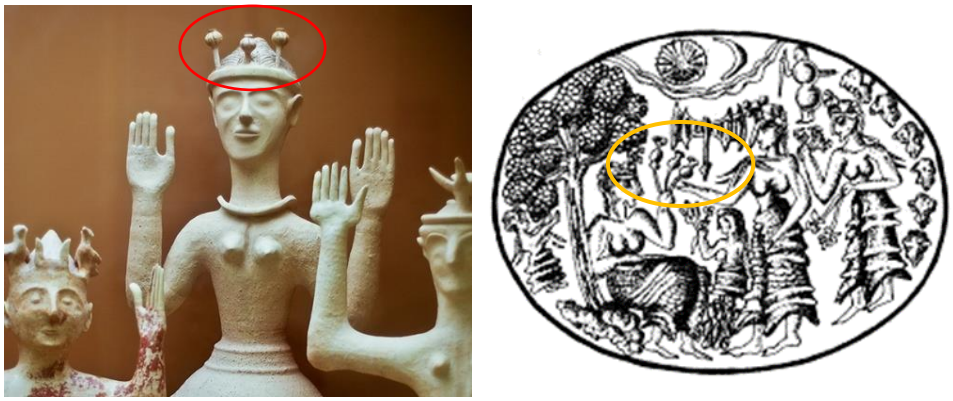

Figure 1.1 Opium poppy capsules on Hittite sculptures and Emirdag coin

Poppy word was called as “haşşika” by Hittites (around Bogazkoy in Turkey), means to sleep or to relax. It is very intriguing smilarity in Turkish using as “haşhaş”. (Baytop, 1963; Ertem, 1974). Opium poppy was a very important export material during the Ottoman Empire. Opium were exported to UK and USA respectively 41.130 and 40.800 kg at 1858.

There was no limitation for poppy cultivation in Turkey until 1933 (Gevenkiris, 2011). In 1933, controlled poppy cultivation and production was launched and later on, in 1938 with the establishment of Turkish Grain Board (Toprak Mahsulleri Ofisi-TMO) monopoly authority of narcotic drugs was given to TMO. In 1971 opium poppy cultivation and making opium production have been banned by the government. Opium Alkaloids Plant started its production in 1981 in the district of Bolvadin, Afyonkarahisar in order to utilize traditional poppy crop and to meet legal alkaloid quality requirements of international and domestic markets (TMO, 2013).

There are 700,000 decares cultivation site of opium poppy in Turkey according to UN regulations. These sites are apportioned to 13 cities by Turkish Grain Board and these cultivation site quota couldn’t fulfill because of the climatic reason which is one of the most significant factors (Bulut, 2015).

The opium poppy is considered to be ideally distributed at transient regions between continental and temperate climates. In Turkey, it is raised in inner black sea and western

transitionary regions. It is well-adapted to climatic conditions in Central Europe. It shows very slow primary development, thereby its cultivation in a moist environment is challenging since it promotes weed growth and potential disease agents. Cultivation sites prone to winds are not desired due to risks of downing or stem rupture. The quick warming soils are suitable for its cultivation. During germination and primary development, plant considerably shows more tolerance to the cold. Minimum recorded germination temperature is +3 °C, however opium poppy seeds generally germinate at +4 °C or higher temperatures with optimum soil moisture content. Seeds do not germinate at lower temperatures. Plants demonstrate well development over +4 °C in fall. Opium poppy plants with well root systems and about 6-8 rosette leaves could have a regular winter with no damages. Opium poppy varieties in some countries are resistant to -5°C while native varieties are tolerant to -10°C, even to -20°C under snow. However, frozen soils and severe winters could damage the first leaves and may even cause the whole plant death. The plant cotyledons exposed to lower temperatures due to late sowing and not emerging because of insufficient precipitation, could be harmed from the frost heave and shock in spring. Plants require a temperature of 2-3 degrees for 30-35 days for healthy development and better vernalization (Yoshida et al., 2010). They become weak and thin without well vernalization and rosette formation. Plants enjoy the hot weather and sun particularly in bolting period. The relatively higher temperatures are required for seed development and maturation during flowering and maturing stages. Opium poppy is a long day plant, therefore sites available with long day light and clear weather during vegetation period increase the morphine content in fruits. Plants require a total temperature of 23-27°C during the development period. The temperature at the time of seed development and maturation has crucial impact on yield of the crops and seed oil content. The sunny, dry and warm weather about 18-20°C is good for this period. Nevertheless, severe heat and low relative humidity delay the pollination during flowering period, and consequently causing the seed yield to drop. During maturation period, the suitable temperature is over 20 °C and sunny weathers. All above indicate that regions with harsh winter conditions and low summer temperatures are not ideal for this plants cultivation. Plants require a 600-700 mm of annual rain and 300-400 mm precipitation is ideal to maintain the primary vegetation period until flowering. Cultivation of the opium poppy is positively affected in regions where annual rain fall is

321.7-694.4 mm. In excessively dry soils, a good germination is not possible thereby seeding time should be chosen after spring or autumn rains. However, rain in the time of flowering period adversely affects the pollination and causes the drops in seed yield. In addition, rain also causes the fungal infections and spread, particularly powdery mildew *Peronospora arborescens.* The highly humid and cloudy days could also lead to the disease during vegetation period. It can also cause the opium to leak out. The early morning rains in full blooming period impede the petals opening and cause the petals sticking to capsule on top, this hindering the pollination resulting in poor seed yields. Countries that practice lancing, which is a process of lacerating the capsule with horizontal and longitudinal lines, may suffer a considerable loss of opium harvest if there is an extreme precipitation immediately after the lancing process. Although it could be considered that plant water demand increases all along the flowering period, rain is not desirable while the plant is at maturing stage. In winter without snow cover, there could be high freezing losses due to the heavy winds. The root of opium poppy is a weak since it has a taproot system. The heavy rains and hail falls could perforate the plant leaves badly, and powerful winds after a rain or irrigation could take down the plants, particularly when the capsules are at development stage. In countries where lancing is allowed, strong winds after lancing may cause frictions between the plants which eventually leads to an extensive loss of opium. Besides, powerful winds give rise to seed losses in dehiscent capsule poppies. So, the appropriate cultivation techniques should be applied to avoid the wind damages (2012, 2013a,b; Bernath 1998; Erdurmus and Ones 1990; Incekara 1964; Kadioğlu 2007; Kahraman 2011; Marquard and Malko 2006; Ogretir, 1985).

1.1.3 Opium poppy alkaloids

Opium poppy produce some chemical compounds, among them benzylisoquinoline alkaloids (BIAs) are very important. These compounds are used in medicine like codeine and noscapine as an antitussive, noscapine as an anticancer, and papaverine as an antispasmodic and morphine as an analgesic agent. Dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA) react for to yield (S)-norcoclaurine at beginning of BIA biosynthesis (Ilari et al, 2009). After steps of methylation and hydroxylation, (S)-norcoclaurine is convert into central intermediate S-reticuline (Choi et al., 2002; Ziegler

et al., 2005). 3′-hydroxy-N-methylcoclaurine 4′-O-methyltransferase (4′OMT) is calayse this reaction (Morishige et al., 2000). After that noscapine, morphine and papaverine are derived from S-reticuline as end products with different pathways. Morphine is produced via an epimerization reaction, (S)-reticuline to (R)-reticuline (De-Eknamkul and Zenk, 1992). Biosynthesis of sanguinarine (benzophenanthridine) and noscapine (phthalideisoquinoline) alkaloids are produced by Berberine Bridge Enzyme (BBE) with the conversion of (S)-reticuline to (S)-scoulerine and this is the first committed step (Facchini, 2007).

The concentration of morphine in opium alkaloids ranges of 5-25%. Alkaloid morphine is used as painkiller since it has anesthetic effect. It performs this effect through numbing the pain center in the cerebral cortex. In addition, it makes addiction as a recreational substance. Thus, without doctor supervision,the use of these kind drugs is prohibited due to a variety of hazards (Ustun, 1998; Tanker and Tanker, 2003).The concentration of codeine in opium alkaloids is 0.5-3%.The analgesic effect of codeine is very low and it is a good cough suppressant since it eliminates the cough reflex. It has been used for centuries as a cough medicine and still demanded. It has less-addictive effect compared to morphine (Ustun 1998; Tanker and Tanker 2003). The name “Thebaine” comes from Thebes province in Egypt which used to export opium (Paul, 2002). Its concentration in opium is 0.2-1% (Tanker and Tanker, 2003). It has less analgesic effect. However, the interest for Thebaine has increased in recent years in production of semi-synthetic drugs which have analgesic, cough suppressant and soothing properties (Onmez, 2007). The concentration of noscapine in opium alkaloids is 2-10% (Tanker and Tanker, 2003). Earlier, noscapine was also known as narcotine (Paul, 2002). Its name was changed to noscopine when its cough suppressant properties have been discovered. The cough suppressant properties of noscopine are equivalent to codeine. It is not a pain killer as well as does not have addictive and anesthetic effects. The concentration of papaverine in opium alkaloids is 0.5-1.3% (Tanker and Tanker, 2003). There are uses for medical purposes andit could be also produced synthetically. The anesthetic and pain relief properties are lower (Onmez, 2007). Papaverine relaxes the smooth muscles and relieves the muscles spasms. It is also an ingredient of drugs used in intestinal spasms and gall bladder obstruction (Ustun, 1998, Tanker and Tanker, 2003).

Table1.2 Important alkaloids in opium (Onmez, 2007)

Name	Formula	Inventor	Date
Morphine	$C_{17}H_{19}O_3N$	Sertumer	1816
Narcotine (noscapine)	$C_{22}H_{23}NO_7$	Robiquet	1817
Codein	$C_{18}H_{21}NO_3$	Robiquet	1832
Narceine	$C_{23}H_{27}O_8N$	Pelletier	1832
Thebaine(Paramorphine)	$C_{19}H_{21}NO_3$	Merck	1835
Pseudomorphine	$C_{17}H_{18}O_3N$	Smiles	1835
Papaverine	$C_{20}H_{21}NO_4$	Hesse	1848
Crytopine	$C_{21}H_{25}O_5N$	Hesse	1864
Rhoeadine	$C_{21}H_{21}O_6N$	Hesse	1867
Lantopine (Tylophorinine)	$C_{23}H_{25}O_4N$	Hesse	1870
Meconidine	$C_{21}H_{23}O_4N$	Hesse	1870
Codamine	$C_{20}H_{25}O_4N$	Hesse	1870
Laudanidine	$C_{20}H_{25}NO_4$	Hesse	1870
Laudanosine	$C_{21}H_{27}O_4N$	Hesse	1871
Hydrocotarnine	$C_{12}H_{15}O_3N$	Hesse	1871
Protopine	$C_{20}H_{19}NO_5$	Hesse	1878
Xanthaline	$C_{20}H_{19}NO_5$	Smith	1893
Papaveramin	$C_{21}H_{25}O_6N$	Hesse	1903
Aporeine	$C_{18}H_{16}NO_2$	Pavesi	1905
Neopine(Oxycodone)	$C_{18}H_{21}O_4N$	Smith	1911
Porfidroksine	$C_{19}H_{23}O_4N$	Rakshit	1919
Oxynarcotine	$C_{22}H_{23}NO_8$	Becket and Wright	1876

1.1.4 Seeds of opium poppy and fatty acids

Poppy seeds can be gray-blue, yellow, white, brownish and pink colors. However, white, blue and yellow seeded varieties respectively are the most widely grown populations in

Turkey. Some of the produced poppy seeds are reserved for farmers' needs and the rest are processed in the free market. Seeds are traditionally used for food, bread and pastries. The most important feature of poppy seed is that it has 40-50% fat and 20-30% protein content. The major components of poppy oil are linoleic acid and oleic acid (Kucuk, 1996). It is used in cosmetics, paint industry, painting and soap making because of its quick drying oil. Recently, oil extracted from poppy seed has been also explored for alternative energy purposes as potential biofuel source (Sabanci et al., 2009, Bozan and Temelli, 2003).

Poppy seeds contain high amounts (40-55%) of fat during full maturity period which is locally used in poppy growing areas. Poppy oil is rich in unsaturated fatty acids, especially oleic and linoleic acids, thereby it is considered to be useful for human health. Fatty acids which are composed of a single covalent bond (-C-C-) between carbon-carbon atoms and which are generally solid at room temperature are called saturated fatty acids (Nas et al., 2001). Oils rich in these fatty acids are also called saturated oils. Lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), arachidic acid (C20:0) and behenic acid are the most important saturated fatty acids in vegetable oils. Particularly palmitic and stearic acids are the most common saturated fatty acids in vegetable oils. Saturated fatty acids can be synthesized in human body regardless of fat consumption, since they can be synthesized from molecules produced by the carbohydrate metabolism (Karaca and Aytac, 2007). In various positions on the carbon chain, fatty acids containing one or more covalent double bonds between carbon-carbon are termed unsaturated fatty acids. Fats rich in these fatty acids are also called unsaturated fats. Employing a double bond structure makes unsaturated fatty acids more reactive than saturated fatty acids. This reactivity is increased by the number of double bonds in fatty acid chain (Nas et al., 2001). Unsaturated fats are essential fatty acids for body. They are liquid at room temperature and the vast majority are herbal (Karaca and Aytac, 2007). Fatty acids containing a double bond in their structure are termed monounsaturated fatty acids or monoenoic fatty acids. The most important two members of this group are palmitoleic acid (C16:1) and oleic acid (C18:1). Crude nuts and their oils such as hazelnut, peanut, walnut and almonds, and olive and rapeseed oils contain the high monounsaturated fatty acids (Karaca and Aytac, 2007). Fatty acids containing two or more double bonds are called polyunsaturated fatty acids or polyenoic fatty acids. Linoleic (C18:2), linolenic (C18:3), arachidonic (C20:4),

eicosapentaenoic (C22:5) and docosahexaenoic (C22:6) acids are the most important polyunsaturated fatty acids. The composition of fatty acids in oil plants is not stable and their synthesis are changed based on the genetic, ecological, morphological, physiological and cultural applications (Baydar, 2000).

Linoleic and linolenic acids are octadecanoic acids containing more than one double bond. These fatty acids, also known as vitamin F are the basic substance for human nutrition. A mixture of unsaturated fatty acids containing linoleic and linolenic acids is obtained from soybean oil and other suitable vegetable oils (Anon., 2012)

Stearic acid is a saturated fatty acid represented by formula $CH_3(CH_2)_{16}COOH$. In most animal and plant-derived solid-liquid oils, it is often found in the form of glyceride stearin. Stearic acid and its compounds, in particular salts (stearates), are of commercial importance. Long chain alcohol esters are known as waxes. The esters of other monohydric and polyhydric alcohols are used in manufacture of varnishes and in production of nonionic surfactants. Alkali metal salts are soluble in water. These are the main ingredients of toilet and laundry soaps, such as oleates and palmitates. Metallic stearates are found in the composition of petroleum grease. Zinc stearates are used in cosmetics, paints (other metal salts), pharmaceuticals and fungicides. Stearic acid amides are used in the manufacture of waterproof fabrics (Anon., 2012).

Poppy is a versatile plant that is used as food for its seeds and oil, animal feed for its oil cake, and medicinal purposes for alkaloids contained in capsules. The fat content in seeds varies according to the color but within the limits of 42-58%. The oil from seeds is a high quality edible oil (Erdurmus and Ones, 1990). Its content has an average of 11.0% palmitic, 0.4% palmitoleic, 1.9% stearic, 15.0% oleic, 71.3% linoleic and 0.6% linolenic fatty acids (Atakisi, 1999). Poppy oil cake is a precious animal food and contains an average of 36% crude protein and 12% crude oil. The fat rate in the milk of dairy animals fed with poppy oil cakes has been reported to be increased (Erdurmus and Ones, 1990).

Oil extracted from poppy seeds is used in the kitchen in salads and as frying oil. Because it is from semi-drying oils, it is extensively used in painting especially in fine arts, and in soap, perfume, cosmetics and other branches of industry (Incekara, 1964; Bruneton, 1995; Krishnammarg, 1996; Nemeth, 2002; Arslan et al., 2011a). It can be a renewable resource

to provide valuable components such as tocopherols and phytochemicals. It is also known as an important source of dietary regimes due to the tocopherol and fatty acids. In many countries, it is also used as additive in baby and other foods because of tocopherols (Oomah and Mazza, 1999; Peter, 2001; Lampi et al., 2002; Moyad, 2005). Poppy oil is also used in treatment of diarrhea, bloody diarrhea and burns. Poppy seeds are good source of energy due to the high protein and mineral contents (Tetenyi, 1997). In rare, poppy seeds in some foods may cause the allergic reactions. People allergic to the snacks such as hazelnut and walnut, and pollens may demonstrate such a reaction after eating the poppy seeds (Keskin and Sekerel, 2006). A considerable amount of poppy oil was produced in France and Germany for use in food in Europe in the late 18th century and earlier first half of 19th century. Later, poppy's value and industrial demand for its oil were decreased (Bryant, 1988). The remaining components -after oil-extraction- such as protein, carbohydrates and phenolics (a non-nutrient biologically active substance) are considered to be an important source (Naczk and Shahidi, 2006). The oil cake rich in protein, fat and nitrogen-free ingredients is a useful animal feed, especially for cattle and buffalo. The famous Afyon cream is produced from the milk of these animals. Poppy capsules are widely used as an arrangement in dry floriculture (Arslan et al., 1986; Arslan et al., 2011). The seeds can be roasted as snacks and be used in cake decoration and muffins. The leaves can be used in salads during the first developmental periods. The straws are used as wood fuel in areas with low fuelwood (100-600 kg straws can be obtained from one hectare in relevant conditions) (Er and Arslan 1972; Camcı, 1983). Alternative fuels have begun to be developed due to the reasons such as decrease in oil reserves, and rapid increase in prices and air pollution. One of the alternative fuels investigated for diesel engines is vegetable oils (Altun and Gur, 2005). The poppy oil may be used as fuel for diesel engines in future. Today, poppy is the most important medical plant in Turkey, and it has a big share in the country’s economy because Turkey continuously supplies the quality products (alkaloids and seeds) to the world markets. Poppy agriculture is legally under the control of United Nations in countries such as Turkey, India, Australia, France, Spain, Hungary, Czech Republic and China. It is illegally cultivated in some Asian countries particularly in Afghanistan, as well as in Mexico and South America. The United Nations has accepted Turkey and India as “traditional poppy producer” countries (Arslan et al., 1986; Ehleringer et al., 1999).

Turkey has more than 50% of the legal poppy cultivation areas. Turkey's soil and climatic conditions are very suitable for poppy cultivation to obtain abundant and especially high quality products. For this reason, poppy is a culture plant in Turkey grown for its seed and opium (Emiroglu, 1978).

Table 1.3 List of satured fatty acids

Common Name	Systematic Name	Structural Formula	Lipid Numbers
Propionic acid	Propanoic acid	CH_3CH_2COOH	C3:0
Butyric acid	Butanoic acid	$CH_3(CH_2)_2COOH$	C4:0
Valeric acid	Pentanoic acid	$CH_3(CH_2)_3COOH$	C5:0
Caproic acid	Hexanoic acid	$CH_3(CH_2)_4COOH$	C6:0
Enanthic acid	Heptanoic acid	$CH_3(CH_2)_5COOH$	C7:0
Caprylic acid	Octanoic acid	$CH_3(CH_2)_6COOH$	C8:0
Pelargonic acid	Nonanoic acid	$CH_3(CH_2)_7COOH$	C9:0
Capric acid	Decanoic acid	$CH_3(CH_2)_8COOH$	C10:0
Undecylic acid	Undecanoic acid	$CH_3(CH_2)_9COOH$	C11:0
Lauric acid	Dodecanoic acid	$CH_3(CH_2)_{10}COOH$	C12:0
Tridecylic acid	Tridecanoic acid	$CH_3(CH_2)_{11}COOH$	C13:0
Myristic acid	Tetradecanoic acid	$CH_3(CH_2)_{12}COOH$	C14:0
Pentadecylic acid	Pentadecanoic acid	$CH_3(CH_2)_{13}COOH$	C15:0
Palmitic acid	Hexadecanoic acid	$CH_3(CH_2)_{14}COOH$	C16:0
Margaric acid	Heptadecanoic acid	$CH_3(CH_2)_{15}COOH$	C17:0
Stearic acid	Octadecanoic acid	$CH_3(CH_2)_{16}COOH$	C18:0
Nonadecylic acid	Nonadecanoic acid	$CH_3(CH_2)_{17}COOH$	C19:0
Arachidic acid	Eicosanoic acid	$CH_3(CH_2)_{18}COOH$	C20:0
Heneicosylic acid	Heneicosanoic acid	$CH_3(CH_2)_{19}COOH$	C21:0
Behenic acid	Docosanoic acid	$CH_3(CH_2)_{20}COOH$	C22:0
Tricosylic acid	Tricosanoic acid	$CH_3(CH_2)_{21}COOH$	C23:0
Lignoceric acid	Tetracosanoic acid	$CH_3(CH_2)_{22}COOH$	C24:0
Pentacosylic acid	Pentacosanoic acid	$CH_3(CH_2)_{23}COOH$	C25:0
Cerotic acid	Hexacosanoic acid	$CH_3(CH_2)_{24}COOH$	C26:0
Heptacosylic acid	Heptacosanoic acid	$CH_3(CH_2)_{25}COOH$	C27:0
Montanic acid	Octacosanoic acid	$CH_3(CH_2)_{26}COOH$	C28:0
Nonacosylic acid	Nonacosanoic acid	$CH_3(CH_2)_{27}COOH$	C29:0
Melissic acid	Triacontanoic acid	$CH_3(CH_2)_{28}COOH$	C30:0
Henatriacontylic acid	Henatriacontanoic acid	$CH_3(CH_2)_{29}COOH$	C31:0
Lacceroic acid	Dotriacontanoic acid	$CH_3(CH_2)_{30}COOH$	C32:0
Psyllic acid	Tritriacontanoic acid	$CH_3(CH_2)_{31}COOH$	C33:0
Geddic acid	Tetratriacontanoic acid	$CH_3(CH_2)_{32}COOH$	C34:0
Ceroplastic acid	Pentatriacontanoic acid	$CH_3(CH_2)_{33}COOH$	C35:0
Hexatriacontylic acid	Hexatriacontanoic acid	$CH_3(CH_2)_{34}COOH$	C36:0
Heptatriacontanoic acid	Heptatriacontanoic acid	$CH_3(CH_2)_{35}COOH$	C37:0
Octatriacontanoic acid	Octatriacontanoic acid	$CH_3(CH_2)_{36}COOH$	C38:0

1.1.5 Element contents of opium poppy seeds

A known anti-bacterial element, silver (Ag) is also a K uptake inhibitor (Hendrix and Higinbotham, 1974). Even though Ag has proven toxic effects, there are some evidences that Ag has promoting effect on grass root growth (Ozoliniya, 1986) and seed germination (Hojjat and Hojjat, 2015).

Aluminum (Al), the third ample mineral in lithosphere which is also a heavy metal, has an amphoteric character that allows reactions with strong alkalis and acids and mainly found as aluminium hydroxide in soil. There is inadequate data about metabolical role of Al even in Al-accumulating plant species (Schmitt et al., 2016), however, some Al-resistant plants can use low levels of Al to accumulate other minerals effectively (Clark, 1977; Foy, 1978) and to control permeability of cell membrane (Slaski, 1994). On the other hand, it is clear that high Al concentrations causes toxicity that leads to delayed germination (Nosko et al., 1988; Machado et al., 2015), reduced root length, low cell division rates, and cation imbalance in plants (Foy, 1978).

Antimony (Sb) is a non-essential element and can promote auxin synthesis by rhizobacteria (Picard and Bosco, 2006). There is no data about Sb toxicity in plants as well as Barium.

Arsenic (As) is a bioaccumulative heavy metal passively taken up with water flow by plants (Thoresby and Thornton, 1979). It is widely known that as toxicity is lethal even in low concentrations as it inhibits ATP synthesis and causes hypermethylation of important genes of all organisms except some chemosynthetic bacteria (Hughes, 2002). Consequently, cells exposed to high levels of as cannot even initiate apoptosis due to low ATP, thus, necrotic tissues occurs all over the plant especially in roots. Nevertheless, it is reported that, low concentrations of as may induce seed germination (Li et al., 2007).

B takes part in almost all metabolically reactions that leads to plant development. In addition, B is also induces pollen germination (Gupta and Solanki, 2013). Although B is an essential for many plants, exceeding amounts of B cause necrosis in all plant parts.

Beryllium (Be) is the lightest alkaline element found in volcanic and clayish soils as well as alkaline rocks (Kabata-Pendias, 2011) and is used in high technology products such as

nuclear weapons, aircrafts and X-ray machines. Due to high production rates and improvements of these industries, Be is becoming a major soil pollutant. It is reported that Be levels between 2 to 16 mg/kg is highly toxic for plants (Gettier and Adriano, 1987) which causes dry weight, root length and shoot length reductions as well as reduction of seed germination (Kaplan et al., 1990).

Cadmium (Cd) is a non-essential mineral for plants and a major pollutant (Järup, 2003). Cd can prevail in soil for a long time that makes Cd very harmful for especially aquatic ecosystems. Long term Cd can cause kidney failure and lung cancer in mammals.

Calcium (Ca) is major macro element as it participate in several plant structures such as cell wall and plasma membrane. Additionally, Ca has an important role on ion regulation, membrane permeability and stomatal regulation (Hawkesford et. al., 2011).

Chromium (Cr) compounds also is a dangerous especially at low pH for all type of ecosystems. High concentrations of Cr stunts plant development by inhibiting chlorophyll synthesis, causing mineral deficiencies, harming roots and bringing about reactive oxygen species (Yadav, 2010).

Cobalt (Co) is one of the most toxic heavy metals due to its high competition with other minerals. However, effects of Co are not fully understood as these effects are highly dependent on concentration and plant species. Even in low concentrations, Co inhibits germination of *Pennisetum americanum* L. and *Parkinsonia aculeata* L. (Burhan et al., 2001) while increasing germination rate, dry weight, root and shoot length of *Eleusine coracana* L. and *Oryza sativa* L. at 5 ppm concentration (Jayakumar et al., 2008). Co also increases N_2 fixation rates of some bacteria, algae and higher plants such as legumes (Nicholas, 1975).

Copper (Cu) is compound of crucial enzymes such as catalase and peroxidase that makes them essential. Cu is also takes part in photosynthesis, respiration and water. (Yadav, 2010; Wuana and Okieimen, 2011; Tchounwou et. al. 2012). Excessive Cu accumulation can cause root and photosystem damages while its deficiency leads to remarkable plant growth inhibition (Kabata-Pendias, 2011).

Fe is also a nutrient heavy metal for almost all living organisms as it takes part in energy production in all types of cells (Kabata-Pendias and Mukherjee, 2007). Iron is an important component of hemoglobin and a structural element of mammalian tissues. It is reported that over 400 μg g^{-1} of Fe is toxic for plants that can cause damage by oxidative stress (Becana et al., 1998). Additionally, excessive Fe accumulation can cause several cation deficiencies such as Mg and Ca (Quinet et al., 2012).

Lithium (Li), which is a major soil pollutant, is the lightest metal found in earth and a very reactive element that can easily absorbed by plants. Soil concentration of Li ranges from 13 to 28 mg/kg all around the world (Kabata-Pendias, 2011). There is still no evidence about lithium whether it is a nutrient element or not, however, it is known that Li affects seed germination (Kalinowska et al., 2013) and plant growth (Angino et al. 1974) even in small concentrations due to its toxic effect or by inhibiting nutrient uptakes such as Zn and Mg.

Magnesium (Mg) is relatively the most important mineral element for plants as it is the main component of chlorophyll which is an essential for photosynthesis. Additionally, Mg is an important cofactor for numerous enzymes such as ATPase and RuBP carboxylase (Hawkesford et. al., 2012). High concentrations of Mg can induce ROS synthesis in plants, chlorosis and mineral deficiencies (Fernando and Lynch, 2015).

Manganese (Mn) is also an essential metal participates in structures of several enzymes for instance transferases, lyases, ligases, integrins, phosphotransferase, reverse transcriptase and Mn-superoxidedismutase (Law et al., 1998). However, its excessive amounts has neurotoxic effect on nervous system that leads to chronic nervous and psychiatric disorders (Yin et al., 2010).

Mercury (Hg), one of the most pollutant heavy metals, causes dramatic plant growth inhibition and seed germination by preventing photosynthesis in leaves and K uptake from roots (Hendrix and Higinbotham, 1974).

Molybdenum (Mo) is essential for plants as it is main constituent of enzymes such as nitrate reductase and some oxydases important for N_2 fixation (Kabata-Pendias, 2011).

Nickel (Ni) is an essential trace metal that takes part in different types of reactions such as hydrogenation and oxidation by participating in enzyme structures. Nonetheless, excessive amounts of Ni is toxic for plants as it inhibits activities of crucial enzymes (Wuana and Okieimen, 2011).

Pb is referred as most dangerous pollutant to environment. Pb increases ROS synthesis, malnutrition and causes anatomical and structural decline (Yadav, 2010).

Phosphorus (P) is one of the irreplaceable mineral nutrients for plants, crucial for photosynthesis and respiration. P is also an important component for phospholipids that forms plasma membrane and adenosine-tri-phosphate (ATP). Phosphorus deficiency leads to necrotic tissues, delayed maturation of plantlets, decrease in plant growth and even death.

Potassium (K) is the most abundant macro element in cytosol that regulates ion concentrations, osmosis, membrane potential and plant-water interactions. Furthermore, K involves in CO_2 fixation in photosynthesis, enzyme activation and maintaining electrical potential (Hawkesford et. al., 2012; Benito et al., 2014).

One of the important trace elements, Selenium (Se) is found at low concentrations between 25-100 μg/kg in plants. (Kabata-Pendias, 2011). In the last decade, researches on Se are numerous as it is thought to be an effective anti-oxidant. Se can prevent UV induced stress and relieve oxidative stress in plants (Seppänen et al., 2003). Some plants also over-acumulate Se to protect themselves from animals.

Sodium (Na) is an alkali metal and its compounds are highly water-soluble. In spite of being non-essential for plants, Na can be an important mineral nutrient for some species especially halophytic plants (Maathuis, 2014). Surprisingly, food plants grow in low Na soils are more tasty (Maathuis, 2014).

Strontium (Sr) is an alkaline earth metal that can be easily available in soil due to susceptibility to weathering and has very similar properties with Ca, however, Ca cannot be replaced by Sr in physiological reactions. Sr is not toxic with Ca presence but without suitable Ca concentration Sr can cause plant growth inhibition significantly (Kalingan et al., 2016).

Data on effects of Tin (Sn) are still unclear that there is insufficient data to consider Sn as a nutrient or not. On the other hand, it is known that Sn is toxic to plants and accumulated in roots.

Some trace elements are beneficial at low concentrations and can be used in fertilizers. Titanium (Ti) is a hormetic element that is beneficial for plant growth but not essential (Kuzel et al. 2003). It is proven that low levels of Ti in soil can promote chlorophyll synthesis and, consequently, increases photosynthesis and dry weight (Simon et al. 1988). Additionally, nanosized TiO_2 has a promoting effect on seed germination at low concentrations (Feizi et al., 2012).

Vanadium (V) is another hormetic trace metal concentrated in pitchy sediments and coal (Llorens et al., 2000). Although some algae and bacteria uses V instead of Mo for N_2 fixation as well as hydrogene peroxide catabolism, there is no evidence of neither essentiality nor toxicity for higher plants. It is reported that increasing concentrations of V has no effect on lettuce seed germination, still, germination of following seeds significantly delayed in all V concentrations (Lepp, 1977).

Zn is another important heavy metal for plants for its structural significance and similarity with Mg (Wuana and Okieimen, 2011). Zn joins structure of a wide range of metabolites, enzymes, hormones and transcription factors (Kabata-Pendias and Mukherjee 2007; Ricachenevsky et al., 2015). Excessive amounts of zinc provokes retarded growth, decreases photosynthesis rates and causes mineral deficiencies such as Mn and Cu (Kabata-Pendias and Mukherjee, 2007).

Thallium (Tl) and Urainum (U) are known pollutants and highly toxic to any living organisms.

1.2 Genetic Analyses

1.2.1 Phylogenetic relationships

The classification of species has begun at around 1750s and later it became a separate discipline known as taxonomy. In its earlier phase, taxonomic classification was primarily relied on the anatomical and morphological features of organisms but by the time it was insufficient. So, the development of new tools and methods has been a must. In this sense, introduction of PCR (polymerase chain reaction) and DNA sequencing technologies has revolutionized all biological disciplines including the taxonomy. These new technologies can help identify a specific gene or whole genomic sequence to use in further phylogenetic and taxonomical studies. The logic behind DNA barcoding is also based on these technologies, sequencing of particular DNA regions could be used as identification tool. The microgenomic identification systems, which rely on sequencing of small DNA segments, could allow the species discrimination. Thus, it has found solid grounds in identification of species such as bacteria, protists and viruses that located at morphologically less distinctive group (Hebert et. al., 2003). In terms of simplicity, reliability, reproducibility and stability issues, DNA sequencing has advantages over other conventional PCR-based DNA markers. Many genomic regions including coding and noncoding sites, that have discriminative power, have been identified such as mitochondrial gene cytochrome c oxidase 1 (CO1), nuclear internal transcribed spacer (ITS), matK gene, rpoC1 gene, psbK–psbI spacer, rpoB gene, atpF-atpH spacer, *trnL-trnF* spacer/gene, trnH-psbA spacer and rbcL gene. But selected DNA region used in DNA sequencing has to possess three main features such as universality, discriminative power and sequence quality/coverage. The "universality", selected DNA region must be in genome of the studied species, the "discrimination power", able to discriminate the species from each other, and the "sequence quality/coverage", easily identifiable from both directions (CBOL Group, 2009). For instance, ribosomal RNA genes (rRNA) are better at revealing the ancient relationships in species since they are exposed to small changes over time. Besides, plastid and mitochondrial genes are suitable for identification of divergences in closely related species since they could rapidly change (Hebert et. al., 2004). In animals, a standard marker such as mitochondrial cytochrome c oxidase 1 (CO1) gene has been reported to study the phylogenetic relationship but plants did not have such

a particular marker (Yao et al., 2010). Some candidate genes/regions in plants have been proposed as a possible standard marker but none were commonly accepted by taxonomic community (CBOL Group, 2009). However, nuclear ribosomal DNA (nrDNA) ITS regions have been often used in phylogenetic studies of various life forms. Recently, ITS region has been suggested to be a universal barcode of all fungi (Schoch et al., 2012). Additionally, chloroplastic *trnL- trnF* spacer was also employed in relationship studies of plants and other organisms.

1.2.2 Internal Transcribed Spacer (ITS)

Ribosome is very important cell organelle functioning in protein synthesis. Its catalytic center mainly comprises the structurally and functionally conserved rRNAs. Thus, rRNAs possess high sequence conservation interspersed with variable regions. Besides, ITS and IGS regions between closely-related species also demonstrate the significant divergence. So, exons of rRNA genes can distinguish the distantly-related species while introns (ITS1 and 2) could identify the closely-related species. This makes rRNA cistrons a suitable element for phylogenetic studies. rRNA encoding genes are usually organized in an operon with ITS region which has power to discriminate the closely-related species (Lee et. al., 2009; Porras-Alfaro et. al., 2014). rRNA cistrons could disclose the phylogenetic relationship in prokaryotes such as bacteria, cyanobacteria and archaea, and eukaryotes such as animals, plants and fungi (Hebert et. al., 2004; Gillespie et. al., 2006; CBOL Group, 2009; Lee et. al., 2009). In eukaryotes, rRNA cistrons are arranged at nucleolus organizer region (NOR) which contains tandem repeats of ribosomal genes. Eukaryotic rRNA cistrons include ITS1, ITS2, 5.8S, 18S and 28S regions. Of these, ITS1 and 2 introns, and 5.8S gene are collectively known as ITS region. ITS1 and ITS2 regions are spliced following transcription (Schoch et. al., 2012).

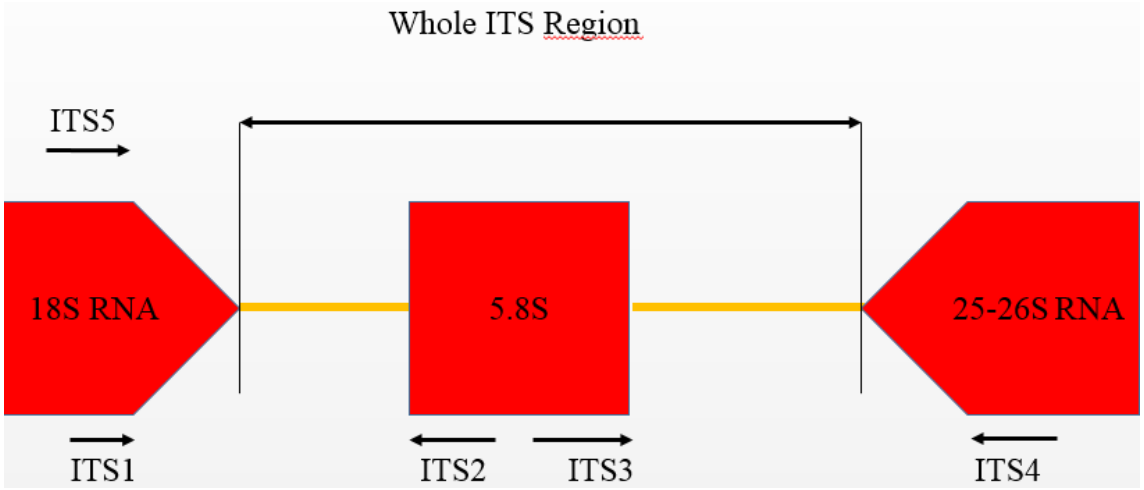

Figure 1.2 Structure of ITS region on nuclear DNA (modified from Porras-Alfaro et al., 2014).

1.2.3 Chloroplastic *trnL-trnF* spacer as phylogenetic marker

The introduction of DNA sequencing technologies provided an impetus in molecular biology in search of comparative genome analyses, phylogenetic studies, mutation analyses, genome mapping and gene annotation. Upon completion of first genome sequencing (Fleischmann et al., 1995), many other organisms have been queued.

The chloroplasts primarily function in photosynthesis to produce glucose, starch, fatty acids, amino acids and pigments. Based on their roles, chloroplastic genes can be classified in three main groups such as ribosomal protein genes, photosynthetic genes and other genes with different functions (Xu et al., 2015). Compared to nuclear genomes, chloroplastic genomes (cpDNA) have more conserved structure with a quadripartite organization including a small single copy (SSC) and a large single copy (LSC) region, and two large inverted repeats (IR). cpDNAs are also highly conserved in terms of structural organization, size and gene order, and relatively resistant to major INDELs, inversions transpositions and SNPs (single nucleotide polymorphism). So, chloroplastic genomes are suitable for phylogenetic studies. The chloroplast DNA is ample due to genome polyploidy, in one chloroplast about with 50 copies and considering the availability of 50 chloroplasts in a cell making 2500 cpDNA copies per plant cell (Alzohairy et al., 2015; Schwarz et al., 2015). In some species, this number could be even more. Currently (as of 2016), NCBI genome database harbored about 890 land plant

chloroplast genomes (ncbi.nlm.nih.gov/genome). Most land plant plastomes were reported to range of 110-170 kb with average of 154 kb (Schwarz et al., 2015). The chloroplastic coding and noncoding regions have been often employed in phylogenetic and taxonomic works, especially in analyses of basal clades with low mutation rates. Generally, cpDNA is uniparentally inherited as single copy, paternally in gymnosperms and maternally in angiosperms. In addition, it is non-recombinant even though nuclear genes are with at least two copies, which may show up with gene conversion and recombination (Soltis and Soltis, 1998; Small et al., 1998; Guo et al., 2007). In phylogenetic studies, it was demonstrated that noncoding regions are more powerful in identification of lower taxonomic ranks due to their absence of functional restrains. Thus, these regions could phylogenetically ensure more distinctive and informative features (Small et al., 1998). During protein synthesis, tRNAs recognize the specific codons on mRNA and then transfer the particular amino acids into the protein-building machinery. So, tRNAs are required to be produced in large quantities and also highly controlled in response to cellular needs. tRNA genes (*trn*) are also highly conserved in chloroplast genomes. These conserved regions such as trn genes, intergenic spacers and introns are frequently used in phylogenetic studies. The *trnL* (UAA) - *trnF* (GAA) and *trnT* (UGU) - *trnL* (UAA) regions were the first characterized spacers (Taberlet et al., 1991). The chloroplastic trnL gene encodes a tRNA which carries the leucine while *trnF* encodes a tRNA carrying the phenylalanine amino acid. In many studies, *trnL* (UAA) - *trnF* (GAA) genes, and their intergenic spacer and intron regions have been demonstrated to be used in phylogenetic studies (Bohle et al., 1994; Gielly and Taberlet, 1994; Ham et al., 1994; Mes and Hart, 1994: Sang et. al., 1997).

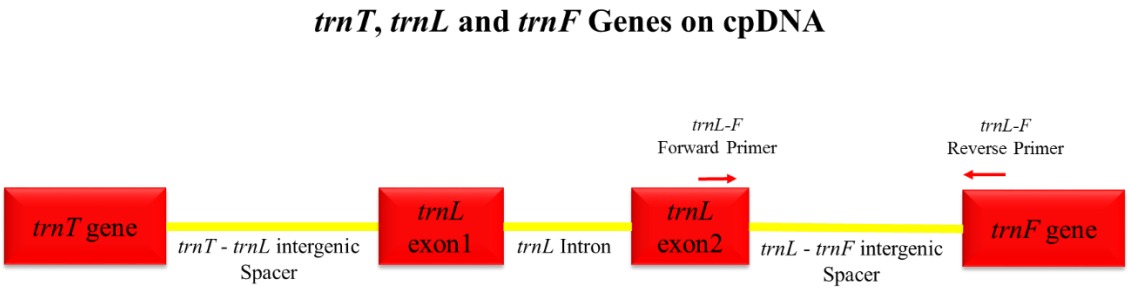

Figure 1.3 The schematic representation of *trnL*, *trnF* and *trnT* genes on cpDNA

Two intergenic spacers separate the *trnT, trnL and trnF* genes, and trnL intron is localized between exon 1 and exon 2 of trnL (UAA) gene. The *trnL-trnF* spacer separates the trnL (UAA) exon 2 from *trnF* (Poczai and Hyvonen, 2011). It has been reported that noncoding regions demonstrate the high genetic diversity, thus sequencing of these regions possess great importance as intraspecific genetic markers in phylogenetic studies (Taberlet et al., 1991). Thus, these regions are genetically more variant due to high mutation rates and lack of gene rearrangements, being potential candidates for studies aiming to discriminate the closely-related species in lower taxa.

1.2.4 Molecular/Genetic Markers and DNA Fingerprinting

Molecular or genetic markers are DNA fragments that match with a particular sequence in the genome. They are frequently used in molecular biology to identify/amplify certain sequence in a given genome. In molecular studies, comparative methods are applied either direct or indirect way. For example, the comparison of sequence/s is a direct method while comparison of amplified fragments is an indirect method. Both approaches are actively used in molecular studies (Avise, 2012). Molecular markers despite their large diversity can be mainly classified in three categories such as DNA sequence polymorphisms, DNA repeat variations and protein variants (allozymes). They have broad range of application areas including evolutionary/phylogenetic studies, genetic diversity analysis, paternity testing and forensic applications, population genetics, molecular linkage maps, marker aided selection, map-based gene cloning, gene tagging and genome mapping (Maheswaran, 2004; Schlotterer, 2004). The first molecular markers are the allozymes which started to be used in mids of 1960s and various other marker types have been developed later. The data from patterns in protein gel electrophoresis was employed as molecular markers, indirectly demonstrating the DNA variations. In mids of 1970s, first DNA sequences and DNA-based molecular markers have been introduced to scientific community. RFLP was the first molecular marker introduced and then various others have been invented (Schlotterer, 2004).

Second generation of DNA molecular markers were mainly based on microsatellites and depended on PCR technology. Microsatellites are arrangements of tandemly repeated DNA sequences (2 - 5 nucleotide) dispersed throughout the eukaryotic genomes. In table below, it should be noted that random amplified polymorphic DNA (RAPD) was among

the most frequently used molecular marker in time line (Williams et al., 1990). In principle, RAPD primers can produce large number of DNA fragments since they attach the multiple sites in genome. In addition, this method does not require a prior knowledge in primer construction (Maheswaran, 2004; Schlotterer, 2004).

The new generation molecular markers are considered to be more effective in terms of easy application, lower cost, reliable results, reproducibility and performance. In these markers, amplified fragment length polymorphism (AFLP) and inter simple sequence repeats (ISSR) were the most used markers in this category. PCR amplification in both markers produce multiple bands, showing a presence/absence variation in individuals. New generation markers include genome targeting, DNA sequencing technologies and new functional markers (Poczai et al., 2013). In addition to them, new kind of markers have been also reported by Poczai et al. (2013), including transposable element based markers (IRAP, REMAP, ISAP, IPBS and SSAP), resistance-gene based markers (RGAP and NBS profiling), targeted fingerprinting markers (DALP, PAAP, SRAP, TRAP, CoRAP and SCoT), conserved DNA and gene family based markers (CDDP, PBA, TBP and ITP), and RNA-based markers (iSNAP, cDNA-AFLP, cDNA-RFLP and EST-SSR). They also have their own advantages or disadvantages based on case or marker type. It was also reported that as of 2012 use of these new markers is about 11% while use of RAPD, ISSR, AFLP and derivate of these markers is about 89% (Poczai et al., 2013).

Moreover, these commonly used markers (ISSR, RAPD and AFLP) have been also reported to have some weakness such as (1) in different samples co-movement of same size fragments from independent loci, (2) co-movement of paralogous bands (3) nested priming, leading to amplicons from overlapped fragments, (4) heteroduplex formation, alternate allelic sequences and/or similar duplicate loci produce products, (5) collision, two or more different fragments of equal size occur in a single lane, (6) non-independence, a band is calculated more than one due to nested priming or codominancy, and (7) artifactual segregation distortions, caused by undetected bad gel resolution false loci scoring or codominancy.

Under constantly changing environment, one of the most important factors for survival of organisms is their genetic diversity. Species are able to maintain internal regulations and adapt to new external conditions thanks to long term genetic diversity. In addition,

knowledge from genetic diversity studies may be also employed to the development of breeding, genetic resource management and conservation strategies. Molecular markers help varieties to genetically improve by aiding their phenotypic selection. They also have a power to resolve which variety suitable for large scale cultivation (Badfar-Chaleshtori et al., 2012).

To measure cultivar identification and genetic variability, various molecular markers and DNA fingerprinting techniques have been introduced. From these markers, AFLP and RFLP have been widely used in studies of genetic relationship. However, their applications are costly and also require expertise. Besides, ISSR and RAPD markers are cheap and easily applicable, for ISSR markers less amounts of DNA is sufficient to give reproducible, highly polymorphic and informative results (Zietkiewicz et al., 1994; Gajera et al., 2010; Sozen, 2010). ISSR is a PCR-based application relying on the amplification of DNA segments between two identical microsatellite regions from opposite directions. ISSR primers are generated from microsatellites and anchored from 5' and 3' as forward and reverse directions. In this method, a prior knowledge is not required for primer design and a single primer could attach to multiple genomic loci. This property of ISSR makes it advantageous over others (Reddy et al., 2002; Huang et al., 2012).

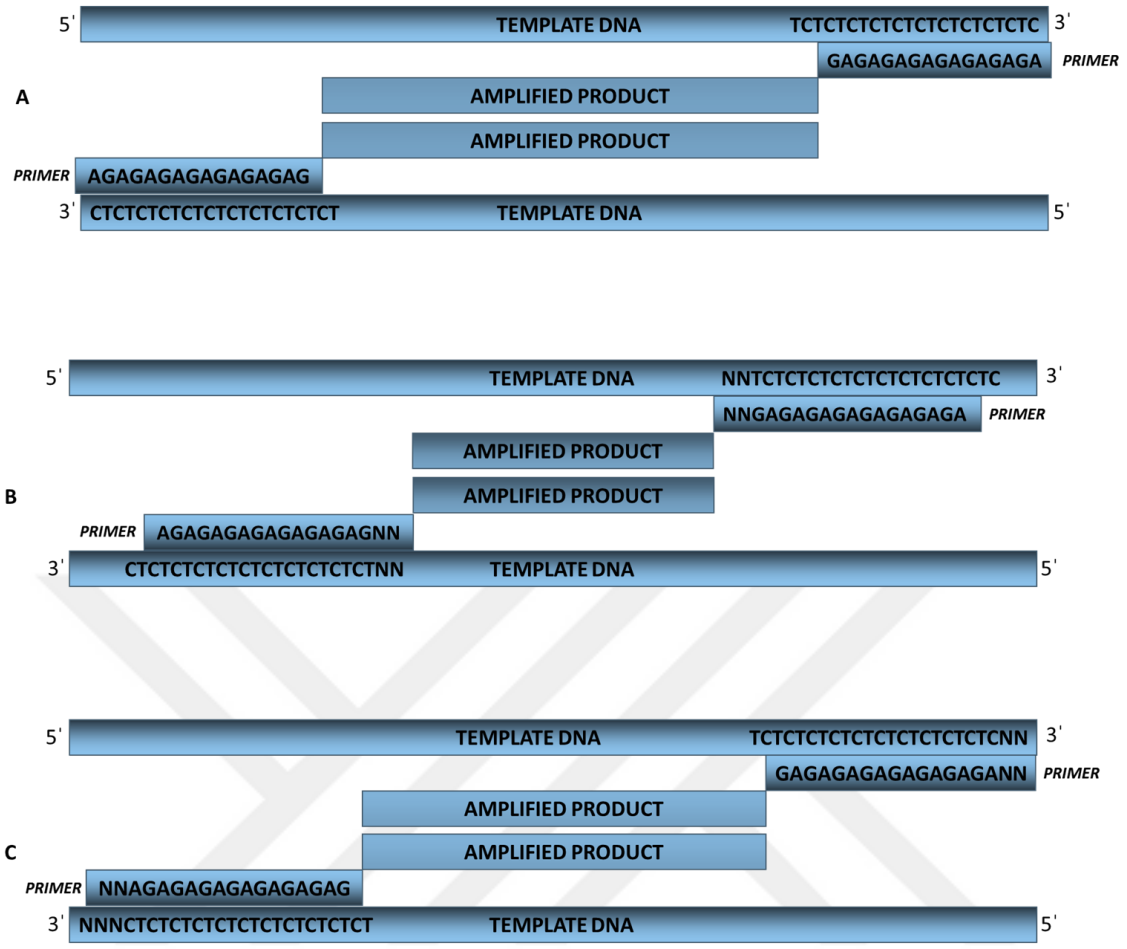

Figure 1.4 Potential matches of primers-template DNA (modified from Reddy et al., 2002). Unanchored (A), 3'-anchored (B) and 5'-anchored (C)

ISSR primers could be employed as unanchored or anchored (1-4 bases) stretched into flanking sequences. Unanchored primers only consist of di-, tri-, tetra- or penta-nucleotides of microsatellite sequences. They also cause slippage and smear band formations since they are attached at everywhere in microsatellite region of target DNA (Figure 1.4 A). Besides, anchored primers consist of microsatellite sequences (2-5 bases flanking sequences). And they attach to specific region on target DNA from both directions and produce more clear bands (Figure 1.4 B and C). Usually, ISSR marker bands vary of 200-2000 bp in length (Reddy et al., 2002).

ISSR is a very simple, quick and highly polymorphic method bearing the advantages of SSRs and AFLP, and universality of RAPD. ISSR markers could be employed in phylogenetic analysis, genetic diversity and genome mapping, genomic fingerprinting,

and in determination of SSR motif frequency (Reddy et al., 2002). The advantages of ISSR markers over others could be listed as relatively low cost, easy application, no need prior knowledge, universality, diversity and discrimination power, and reproducibility (Zietkiewicz et al., 1994; Reddy et al., 2002; Cao et al., 2006).

2. MATERIAL AND METHODS

2.1 Methodology

Genetic analyses were conducted on plants grown in laboratory conditions using certified cultivar seeds and chemical analyses were carried out on the seeds of these plants. Another purpose of this study is to investigate the genetic diversity in plant genomes using ISSR-PCR method. Furthermore, oil, mineral compounds analysis total protein content were employed for chemotaxonomic classification. Finally, this study investigated the genetic relationships between *P.somniferum* varieties by using internal transcribed spacer 1 (ITS1) and *trnL-trnF* intergenic spacer sequences. Mineral nutrient elements and fatty acids in seeds were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) and Gas Chromotography-Flame GC-FID respectively.

2.2 Sampling

Certified papaver cultivar seeds obtained from Turkish Grain Board (Toprak Mahsülleri Ofisi, TMO) Afyon Opium Alkaloids Factory were sown into vials prepared in Plant Molecular Biology and Biotechnology Laboratory of Marmara University, Faculty of Science and Letters. The plants were harvested after observing a sufficient growth for analyses. Young leaf samples from plants were selected for genetic analysis and dry seeds were used for mineral nutrient elements, fatty acids and other chemical compounds such as protein analyses.

2.3 Analysis of Chemical Composition

2.3.1 Sample preparation

The seeds were cleaned from other macro spalls and particles. Then the seeds were preserved in an environment without light or moisture. Collected leaf samples from different varieties were packed in aluminum foil separately prior to being stored at -80°C Lexicon II ULT deep freezer (Esco Micro Pte. Ltd. Singapore) until molecular analysis.



Figure 2.1 -80 Ultra deep freezer.

Seeds were desciccated in 80ºC Electro-mag M6040 P oven (Electro-mag, Istanbul, Turkey) for 24 hrs. Before being used for elemental analysis. Then, samples were weighed 0.2 g, ground by using mortar and pestle and transferred into Teflon vessels containing 8 ml 65% HNO_3 (Merck). All seeds samples were wet ashed in a microwave oven (Berghof-MWS2) according to Abu-Samra et. al.(1975). Procedure was carried on for 5 min at 145ºC, 5 min at 165ºC and 20 min at 175ºC. The samples were filtered by using Whatman filters with 1-2 µm mean pore diameter. Final volume was adjusted to 50 ml by adding ultrapure water in volumetric flasks and then kept in falcon tubes until ICP-MS application. 10, 50, 100, 250 and 500 mg/L stock solutions were prepared by using

multi-element stock solutions 1000 mg/L (Merck). Calibration curves were drawn by using stock solutions.



Figure 2.2 Berghoff microwave oven and Teflon vessels

2.3.2 ICP-MS and element readings

ICP-MS has been widely utilized for analyzing trace element quantities in all sorts of materials, as it can rapidly analyze most elements with very low detection limits and relatively simple sample preparation (Chen et al., 2017). Concentrations of some elements such as Ag, Al, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Sb, Se, Sn, Sr, Ti, Tl, U, V in $mg.kg^{-1}$ dry weight (DW) were measured by using ICP-MS. ICP-MS is built up of two parts, one of which is a plasma generator (ICP) and the other is a mass spectrometer. Wet ashed sample solution is sprayed into plasma generated which is generated by applying an intense electromagnetic field onto argon (Ar) gas. Elements become induced and emit a unique radiation of UV and visible light at characteristic wavelengths. An optical spectrometer detects the emission of radiation. The intensity of light emissions indicates the concentration of elements within the solution. 7700 ICP-MS (Agilent Technologies, California, USA) is used detection of mineral elements.



Figure 2.3 Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS).

2.3.3 Soxhlet extraction method

Soxhlet extraction method is an efficient technique commonly used in oil extraction. This method is dependent on the characteristics of the oil and solvent, duration of the reaction between sample and the solvent, temperature of the process, size of the particles and solvent/sample ratio. The seeds were pulverized and extracted with the aid of a Soxhlet extractor by petroleum ether.



Figure 2.4 Soxhlet extractor and fatty acid extraction

2.3.4. GC-FID and fatty acids analyses

Fatty acid samples obtained by Soxhlet extraction were analyzed by GC-FID. GC-FID was employed for fatty acid measurements since fatty acid ratios in seeds are nutritionally important. The fatty acid methyl esters shortly known as FAME, are formed either from free fatty acids (FFA) depending on esterification type, or from bound fatty acids of triacylglycerols (TAGs) which can be identified by capillary gas chromatography. This process allows identification of many types of FAME built up of 6 to 24 carbon units such as saturated, cis- and trans- variants of monounsaturated and polyunsaturated FAME. Analytical columns of definitive length and polarity are used to separate FAME prior to detection by a Flame Ionization Detector (FID).

Gas chromatography (GC) is a technique used for quantitative chemical analysis of compounds with low molecular weight, typically and preferably less than 1.000 Da. Since it becomes harder to vaporize larger compounds. The samples are either already gases or vaporized liquids inside a vial which were sucked by an injector. A carrier gas which can

be either hydrogen or helium can be used as a carrier (mobile phase) in this method. Hydrogen is hazardous but a speedy method which can double the speed of analyzing process, gives sharper peaks and accelerates the separation. However devices that improves the safety of this process are available. As samples pass through a capillary column the volatilized mixture which is analyzed interact with the stationary phase that coats the inner surface of the column. The stationary phase is described as a microscopic layer of a thin film of liquid nature on a solid, inert surface made up of fused silica. Elution occurs at different times as a result of contrasting interaction of distinctive compounds. This retention time of the compound depending on a series of analysis parameters is used to distinguish compounds from one another.

0.1 ml of oil sample is put in a 5 mL screw-top test tube and 2 mL of heptane is added and shaken. 0.2 mL of the methanolic potassium hydroxide solution was then added; the cap fitted with a PTFE (Polytetrafluoroethylene-Teflon) joint is tightened, and shaken vigorously for 30 seconds. The sample was set aside to rest for a while, a stratification occurs and the solution at the upper part of the tube becomes clear. The upmost layer which contains methyl esters was carefully decanted and the heptane solution is now ready for injection into the GC. If an immediate analysis is not planned, the solution can be stored in a refrigerated environment prior to a gas chromatographic analysis, however it’s not recommended to store this solution for more than 12 hours.

Flame-ionization detection (FID) is an attached part of the GC, and considered as a favorable organic compounds detector which detects the amount of carbon atoms in a given sample. A hot flame consisting of hydrogen and air mixture burns the sample after the column, resulting in formation of carbon ions by the combustion. The current from the ions are then measured by electrodes. The overall efficiency of this procedure might be considered as low since approximately only 1 in every 105 carbon atoms produce an ion, however the total number of produced ions is precisely proportional to the amount of carbon in the sample. Since the sample is combusted entirely, FID is considered as a destructive detector.



Figure 2.5 Gas chromatography- Flame-ionization detection (GC-FID)

Table 2.1 GC-FID working conditions

Injector temperature:	250 °C
Detector temperature:	250 °C
Oven temperature:	165 °C (8 min) to 210 °C at 2 °C/min
Carrier gas:	column head pressure, 179 kPa
Total flow:	154.0 mL min^{-1};
Split ratio:	1:100
Injection volume:	1 µl

2.4 Isolation of Total Protein

UV-VIS spectrophotometry was devised to measure total protein concentrations. Initially, phosphate buffer was prepared for protocol by mixing stocks A and B which were prepared as follows:

Stock A: 2.76 g $NaH_2PO_4.2H_2O$ was taken into an erlenmeyer, and filled up to 100 ml with distilled water for 0.2 M solution.

Stock B: 3.56 g $Na_2HPO_4.2H_2O$ was taken into an erlenmeyer, and filled up to 100 ml with distilled water for 0.2 M solution.

Phosphate buffer: 6.4 ml Stock A and 43.6 ml Stock B were mixed and filled up to 100 ml by adding 50 ml distilled water. Eventually molarity of solution was adjusted to 0.1 M and pH was adjusted at 7.7.

Protocol:

1. 0.4 g seeds was ground and homogenized with a pestle in a mortar, which was put in a container filled with ice, with 2 ml phosphate buffer (0.1 M, pH 7.7).
2. Samples were transferred into 2 ml microcentrifuge tubes and labelled.
3. Samples were centrifuged at +4°C and 12.000 rpm for 20 min.
4. Supernatant was transferred into a clean tube as protein source. Exposure of the samples to high temperature and light was avoided during the process.
5. Protein concentration of the 1.5 µl supernatant was measured with OPTIZEN NANO Q Spectrophotometer (Mecasys Corp., Gyeonggi-do, Republic of Korea) at protein measurement mode.



Figure 2.6 OPTIZEN NANO Q Spectrophotometer. dsDNA and protein measurement modes were shown in Figure.

2.5 Molecular Analyses

Several PCR-based methods can be employed for detection of mutations, genetic similarities and phylogenetic relationships. *Papaver* cultivars were analyzed with PCR using ITS and *trnL-trnlF* markers for phylogenetic relationships and ISSR markers for genetic diversity.

2.5.1 DNA isolation

A genetic study starts with the extraction and purification of nucleic acids since the quality and quantity of nucleic acids directly affects the following procedures. It is stated that performance of PCR reactions or cloning studies might be affected by the presence of contaminants (Miller et al., 2002). Choosing the most convenient method from a wide range of DNA isolation methods that have been available, depends on several criteria such as; (i) target nucleic acid, (ii) source organism, (iii) source tissue, (iv) desired results (e.g., yield, purity), and (v) further studies (e.g., PCR, RT-PCR, cloning, cDNA synthesis). Common features of extraction methods are lysis of the cells, inactivation of cellular nucleases and finally separating the nucleic acids from the rest of cellular components.

To obtain an ample amount of good quality DNA, several isolation methods including manual methods and commercial kits have been devised in this study. The consumables and equipments used in genetic analysis are listed as follows; 1-14K refrigerated and unrefrigerated microcentrifuges, (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) WiseTis Homogenizer (Wisd - Witeg Laboratory Equipments, Wertheim, Germany), WiseMix VM-10 vortex mixer (Wisd - Witeg Laboratory Equipments, Wertheim, Germany), Mixer Mill MM 400 homogenizer (Retsch GmbH., Düsseldorf, Germany) mortar and pestle (Isolab, Wertheim Germany), Lexicon II ULT deep freezer (Esco Micro Pte. Ltd. Singapore), -20 °C deepfreezer (Vestel, Manisa, Turkey), +4 °C refrigerator (Arçelik, Istanbul, Turkey) NB9 unstirred water bath (Nüve, Ankara, Turkey), fume hood (Alptek, Istanbul, Turkey), CTAB (cetyltrimethylammonium bromide), Tris HCl, EDTA, PVP chloroform, octanol, isopropanol and ethanol (Marka).

Wizard DNA Purification Kit (Promega Corp. Wisconsin, USA) was used in DNA isolation. Protocol applied as instructed by the manufacturer was as follows;

1. Leaf samples were ground either with mortar and pestle or Mixer Mill MM 400 homogenizer (Retsch GmbH., Düsseldorf, Germany), then 40 mg of homogenized tissue was transferred into 2ml centrifuge tube.
2. 600 µl nuclei lysis solution was added into tube and vortexed.
3. An incubation time of 45-60 minutes applied on the mixture of nuclei lysis solution and leaf tissue.
4. 3 µl RNase solution was added, and the mixture was inverted and incubated for 15 min at 37°C. Subsequently, the mixture was left to cool down in room temperature for 5 min.
5. 200 µl of protein precipitation solution was added to the mixture and vortexed at high speed.
6. Proteins were precipitated via centrifuge at 16000 g.
7. DNA containing supernatant was then transferred into a clean tube containing 600 µl isopropanol at room temperature.
8. Tube was inverted several times carefully.
9. Mixture was centrifuged once more for a duration of 1 minute at 16000 g and room temperature.
10. Supernatant was carefully decanted. 600 µl 70% ethanol at room temperature was added and the tube was inverted several times to wash the DNA.
11. The mixture is centrifuged again for another 1 minute at 16,000g and room temperature. (Steps 10 and 11 were repeated whenever necessary)
12. Ethanol was evaporated for 1 hour in 60°C in vacuum centrifuge (marka model ekle). The remaining DNA pellet was carefully handled to avoid any loss.
13. 100 µl of RNAse free water was added to rehydrate the DNA. Final solution was incubated for 1hr at 65°C or overnight at 4°C. The solution was periodically mixed by gently tapping the tube.
14. Acquired DNA was either stored at 2-8°C for short term or -20°C for long term.

2.5.2 PCR reactions

DNA cloning and sequencing, phylogenetic studies, forensic sciences, gene function diagnostics, paternity testing, detection of pathogens and diagnosis of genetic diseases are some of many areas where PCR applications are used (Bartlett and Stirling, 2003). Aeris Thermal Cycler Model G96 (Esco Micro Pte. Ltd., Singapore) below was used for amplifications in this study. ITS1 region, *trnL- trnF* Intergenic Spacer and Microsatellite Regions (with ISSR primers) were amplified using this device.



Figure 2.7 Thermal cycler

A PCR reaction requires an optimized mixture of compounds at particular conditions. Template DNA, reaction buffer, $MgCl_2$, dNTP mix, Taq polymerase, primer(s) and ultrapure water were the substances used in PCR reactions (Table 2.2).

Table 2.2 Individual PCR reaction compounds and their total volumes

Reaction Compounds	Quantity
Template DNA	20-100ng
Reaction Buffer (10X)	3µl
$MgCl_2$ (25 mM)	3µl
dNTP Mix (25 mM)	0.3µl
Taq DNA polymerase (1µl/5U)	0.2µl (1U)
Primer (10mM)	1.5µl
Ultrapure Water	16µl
TOTAL	**25µl**

Since the quality and quantity of DNA yield is affected by the PCR conditions, these conditions should be optimized conscientiously. ITS, *trnL–trnF* Intergenic Spacer and ISSR PCR reactions should be performed in agreement with the conditions given in Table 2.3. Annealing temperature, length and other characteristics of primers can be changed conforming to the design and use purposes. A total of 18 primers, two of which are for ITS, two for *trnL-trnF* Intergenic Spacer and 14 for ISSR, were herein used in PCR reactions.

Table 2.3 The schematic representation of major steps in PCR reactions

Step		Temperature	Duration
Initial Denaturation		94-95ºC	3-7 min
35-45 cycle	Denaturation	94-95ºC	30-60 sec
	Annealing	Depends on primer	30-60 sec
	Extention	72ºC	1 min/kb
Final Extention		72ºC	10 min
Hold		4ºC	∞ As desired

Two primers, namely universal ITS1 and ITS2 primers were used in amplification of ITS (Internal Transcribed Spacer). These are given in table 2.4. ITS region consists of ITS1, 5.8S rRNA and ITS2 parts. In this study, ITS1 part of ITS region was amplified. During amplification, ITS primers were annealed at 48ºC.

Table 2.4 The sequences of ITS primers (White et al., 1990)

Primer	Sequence	Annealing Temperature
ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	50°C
ITS2	5'-GCTGCGTTCTTCATCGATGC-3'	

trnL-trnF are tRNA genes on chloroplast genome. Amplification of *trnL-trnF* Intergenic Spacer was performed at similar conditions with ITS reactions such as annealing temperature which was adjusted to 48°C. *trnL-trnF* Intergenic primers were designed by Sang et al., (1997).

Table 2.5 The sequences of *trnL-trnF* primers (Sang et al., 1997)

Primer	Sequence	Annealing Temperature
trnL-F Forward	5'-AAAATCGTGAAGGTTCAAGTC-3'	50°C
trnL-F Reverse	5'-GATTTGAACTGGTGACACGAG-3'	

All ISSR primers were derived from microsatellites and two or more nucleotides follow these to design anchored primers. Total of 18 ISSR primers were selected to amplify the microsatellite sites to reveal the genetic similarity and small changes on genome of *P. somniferum*.

Table 2.6 The sequences of ISSR primers

Primer	UBC Code	Sequence	Annealing Temperature
ISSR1	UBC807	AGA GAG AGA GAG AGA GT	48ºC
ISSR2	UBC811	GAG AGA GAG AGA GAG AC	53ºC
ISSR3	UBC817	CAC ACA CAC ACA CAC AA	50ºC
ISSR4	UBC818	CAC ACA CAC ACA CAC AG	53ºC
ISSR5	UBC820	GTG TGT GTG TGT GTG TC	53ºC
ISSR6	UBC823	TCT CTC TCT CTC TCT CC	53ºC
ISSR7	UBC827	ACA CAC ACA CAC ACA CG	53ºC
ISSR8	UBC825	ACA CAC ACA CAC ACA CT	54ºC
ISSR9	UBC848	CAC ACA CAC ACA CAC ARG	56ºC
ISSR10	UBC849	GTG TGT GTG TGT GTG TYA	54ºC
ISSR11	UBC857	ACA CAC ACA CAC ACA CYG	54ºC
ISSR12	UBC842	GAG AGA GAG AGA GAG AYG	56ºC
ISSR13	UBC875	CTA GCT AGC TAG CTA G	59ºC
ISSR14	UBC829	TGT GTG TGT GTG TGT GC	49ºC
ISSR15	UBC844	CTC TCT CTC TCT CTC TRC	56ºC
ISSR16	UBC841	GAG AGA GAG AGA GAG AYC	50 ºC
ISSR17	UBC847	CAC ACA CAC ACA CAC ARC	49 ºC
ISSR18	UBC855	ACA CAC ACA CAC ACA CYT	51 ºC

2.5.3 Gel electrophoresis

Electrophoretic methods have applications in many areas such as biochemistry, genetics, and medical diagnostic and forensic sciences. Gel electrophoresis is used to segregate and visualize amplicons which are end products of PCR. In this study, CS-300V omniPAC MIDI Power Supply (Cleaver Scientific Ltd., Warwickshire United Kingdom) was used as power supply. Agarose (peqGOLD Universal Agarose, VWR International, Darmstadt, Germany) was used in gel electrophoresis as 1.2 g for 100ml, 3 g for 250 ml [1.2% (m/v)]. Other components used in gel preparation included 1X TBE solution (Fisher Scientific, Massachusetts, USA) of 100 ml small size gel or 250 ml big size gel and 2 µ Ethidium bromide (MP Biomedicals, California, USA) for 100 ml or 5 µ for 250 ml.

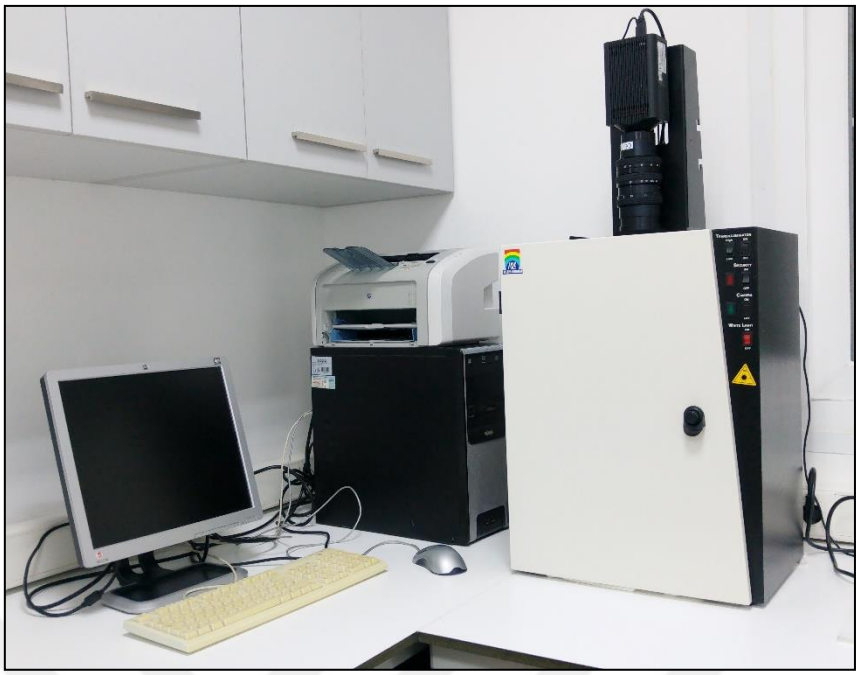

Figure 2.8 UV transilluminator

PCR mixture were mixed with 6X DNA loading dye which contains bromophenol blue and loaded in wells (Termo Fisher Scientific, Massachusetts, USA). Electrophoresis was set up as 90 V and 120 min. After gel electrophoresis, UV transilluminator (Vilber Lourmat, Collégien, France) and WiseUV WUV-L20 UV-Transilluminator (Wisd - Witeg Laboratory Equipments, Wertheim, Germany) were used to visualize DNA bands. ITS and *trnL-trnF* Intergenic spacer amplicons formed single bands whereas ISSR amplicons formed multiple bands.

2.5.4 Genetic diversity analyses

After the gel electrophoresis and visualization, binary codes were assigned as 1 for presence of amplified DNA fragment bands and 0 for the absence of bands. Unclear and ambiguous bands were ignored for scoring and clear and obvious bands are taken into consideration. POPGENE version 1.32 (Yeh et al., 1999) and MVSP 3.22 (Multi-Variate Statistical Package) were used in analysis. Genetic parameters calculated in this study included mean number of observed (Na) alleles, the percentage of polymorphic loci (P), Nei's gene diversity (h), effective (Ne) alleles per locus, (Kimura, 1964) and Shannon's information index (I). Principal component analysis (PCA) test, which was demonstrated with variance-covariance matrix calculated from marker data using MVSP 3.2 program

provided a Graphical representation of ISSR relationships among individuals. A dendogram was generated with Jaccard's similarity coefficients by using unweight pair group method with calculating the arithmetic average (UPGMA) by MVSP 3.2.

2.5.5 Phylogenetic analyses

Single monomorphic bands were generated by ITS and trnL-F Intergenic Spacer primers as expected. To display the relevant bands 3 µ of PCR product and 1 µ of DNA loading dye were mixed together and loaded into 1.2% agarose gel. The rest of the PCR products which were 20-22 µ, were used in sequencing applications. Sequencing processes were conducted by Iontek Molecular Diagnostics Company (IMD - Maslak, Istanbul, Turkey)

ITS1 and *trnL-trnF* Intergenic spacer regions sequencing process were performed for 12 cultivars. Sequenced DNAs were analyzed for short or incorrect DNA, unsuitable sequences were eliminated and the sequencing process was then repeated. Nucleotide data base of GenBank/NCBI (ncbi.nlm.nih.gov/genbank/) was used to obtain DNA sequences of other species. All applications were performed for each ITS1 and *trnl-tnrF* Intergenic Spacer.

ClustalW in BioEdit 7.2.5 (Hall, 1999; Larkin et al., 2007) with default parameters settings was used to gather and align all DNA sequences and MEGA 6 with Maximum-Likelihood (ML) method (Tamura et al., 2013) was employed to construct the phylogenetic tree according to the analysis options given in Figure 2.9. Bootstrap tests were conducted using MEGA 6 with 1000 replicates. Maximum composite likelihood method was used to calculate the evolutionary distances. Datasets were obtained with Kimura 2-parameter nucleotide substitution model and complete deletion option. G+C content (%), Sequence identity matrix, Tajima's test of neutrality (Tajima, 1989), and conserved regions were calculated with BioEdit 7.2.5.

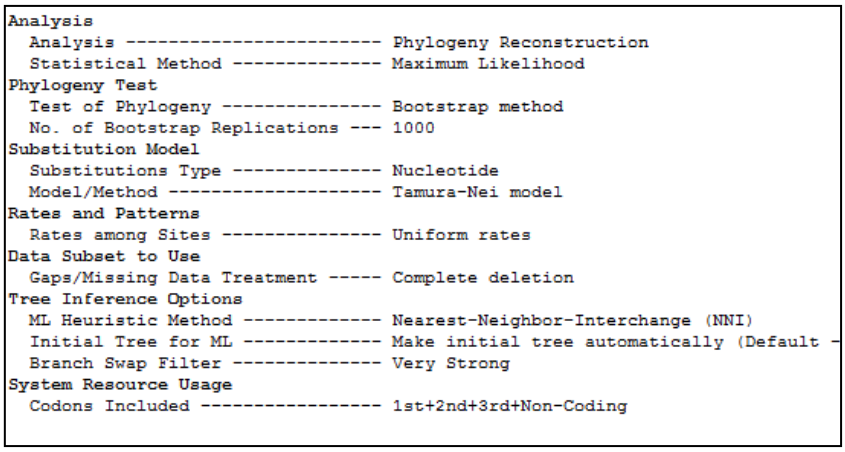

Figure 2.9 The adopted parameters for phylogenetic analysis

2.6 Statistical Analyses

Quantities of all elements, fatty acids and protein amounts were calculated based on the dry weight of samples. The obtained data were analyzed statistically with IBM SPSS Statistics ver. 21 software using hierarchic cluster analysis. Statistical significance was showed as **P<0.01 and *P<0.05.

3. RESULTS AND DISCUSSION

Fatty acids, mineral elements and heavy metal were analyzed to reveal chemotaxonomic relationships. Additionaly, ITS and *trnL-trnF* intergenic spacer sequences and band data of ISSR molecular markers were used for determine the phylogenetic relationships and genetic diversity, respectively.

3.1 Chemical Composition of Opium Poppy Seeds and Statistical Analyses

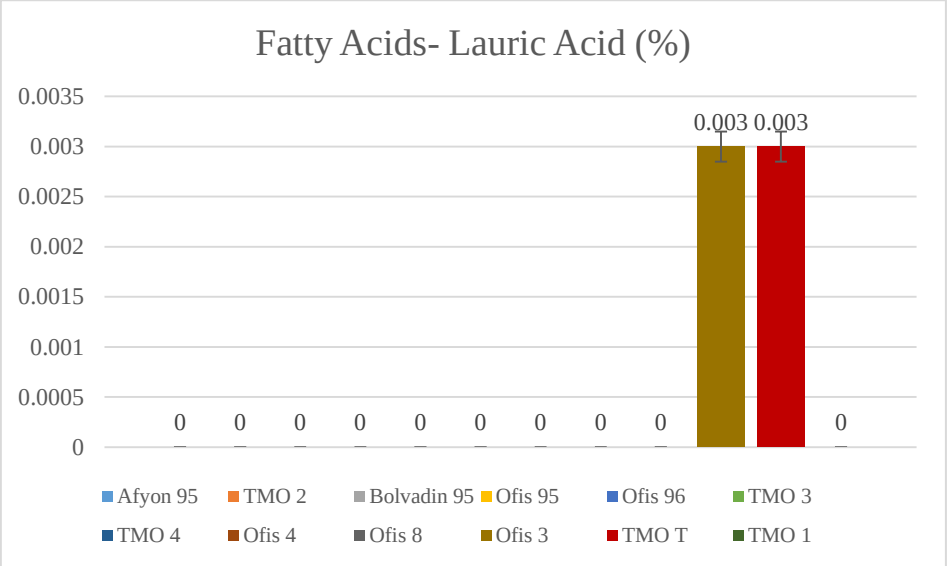

Figure 3.1 Percentage of lauric acid in measured seeds of different *P. somniferum* cultivars

Lauric acid percentage were found to be equal in Ofis 3 and TMO T cultivars. In other cultivars, Lauric acid values were found to be below the measurable limit. Bernath (1998) found lauric acid percentages between 0-13.4% in a study of fatty acids on different poppy variety seeds. The lauric acid values of the cultivars in this study confirm this information.

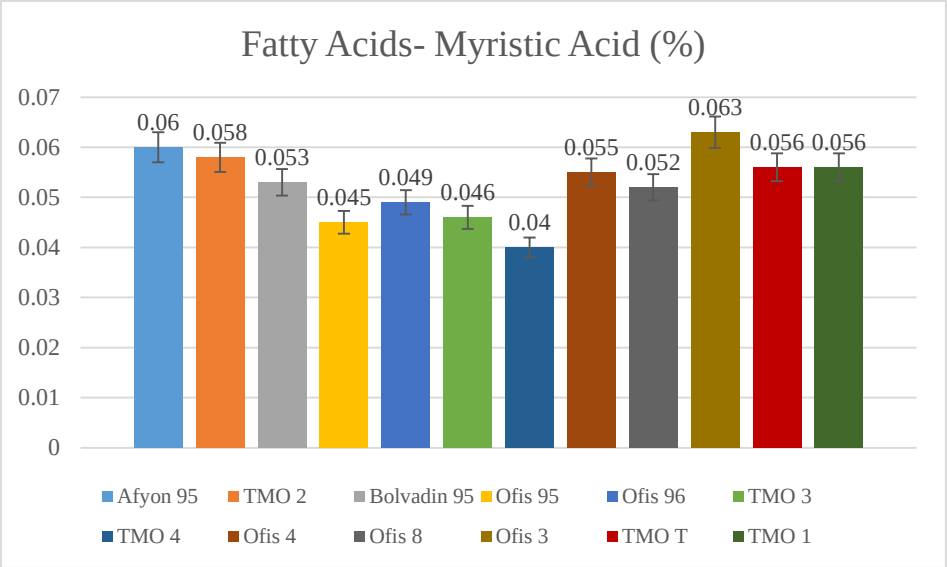

Figure 3.2 Percentage of myristic acid in measured seeds of different *P. somniferum* cultivars

Ofis 3 cultivar have the highest percentage of myristic acid ratios whereas the TMO 4 cultivar have the lowest percentage. Rahimi et al, (2011) determined myristic acid percentages between 0.04-0.06% in different Turkish poppy seed cultivars. TMO 4 percentage is the same in both studies and has the smallest values. Also other values were found to be close to each other in both studies.

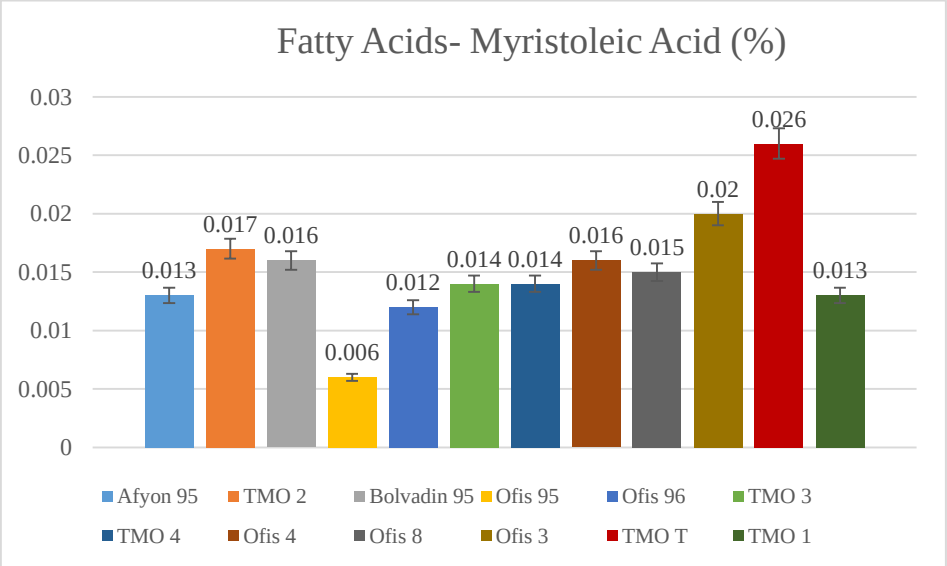

Figure 3.3 Percentage of myristoleic acid in measured seeds of different *P. somniferum* cultivars

Myristoleic acid level of TMO T has the highest (0.026%) rate among all cultivars whereas Ofis 95 has the lowest (0,006%).

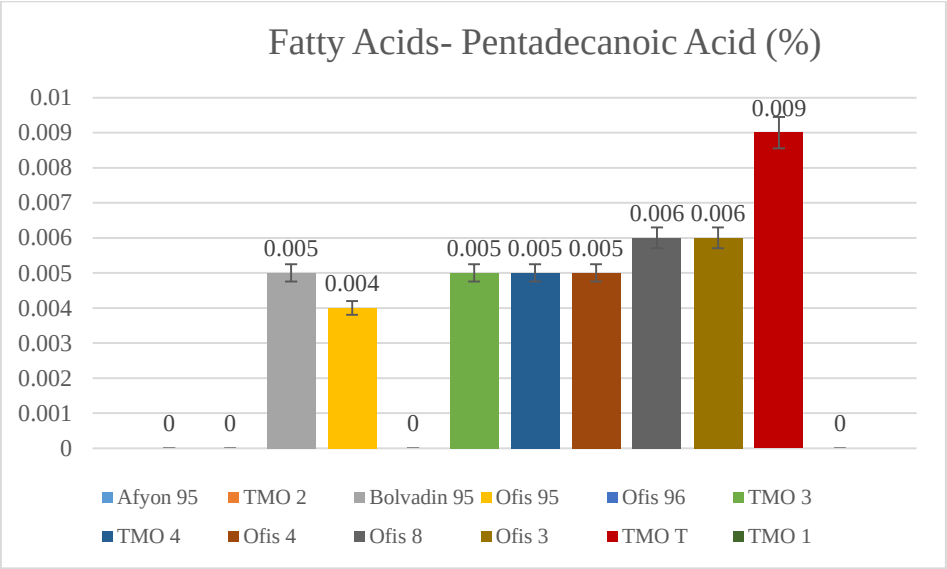

Figure 3.4 Percentage of pentadecanoic acid in measured seeds of different *P. somniferum* cultivars

Pentadecanoic acid amounts were found to be below the measurable value of some cultivars, with TMO T variety at most, Office 8 and Office 3 cultivars at second, followed by TMO 3, TMO 4 and Office 4 cultivars at least in the Office 95 cultivars.

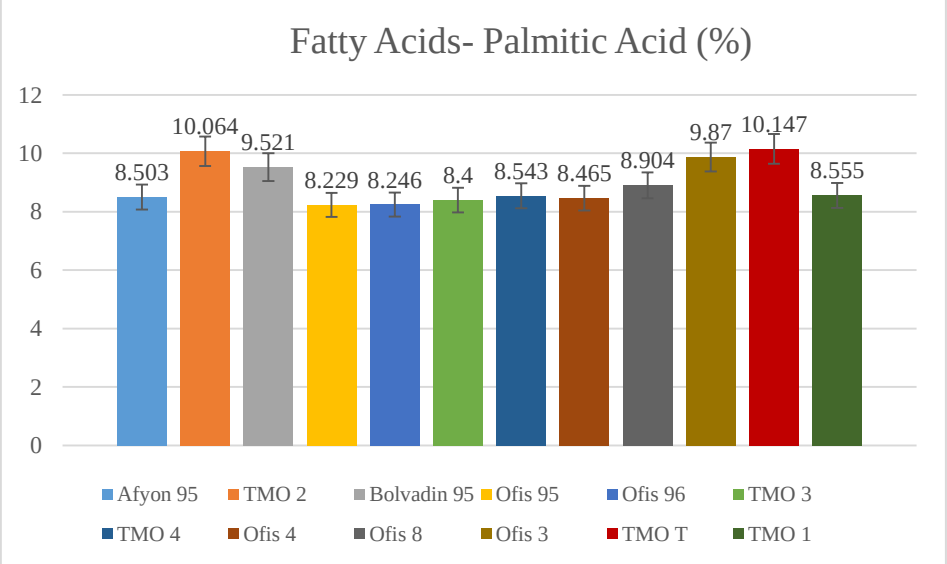

Figure 3.5 Percentage of palmitic acid in measured seeds of different *P. somniferum* cultivars

It is observed that the proportions of palmitic acid contained in the cultivars are close to each other. The highest order is TMO T cultivar and the lowest is Ofis 95 cultivars. Ozcan and Atalay (2006), analyzed palmitic acid percentages between 12.85-18.70% in some opium poppy cultivars seeds. These values were found to be higher than the cultivars studied in the present study. But Rahimi et al, (2011) found between 7.96-10.19% and Bozan and Temelli (2008) found average 9.79% in poppy seeds. These percentages are similar with our study.

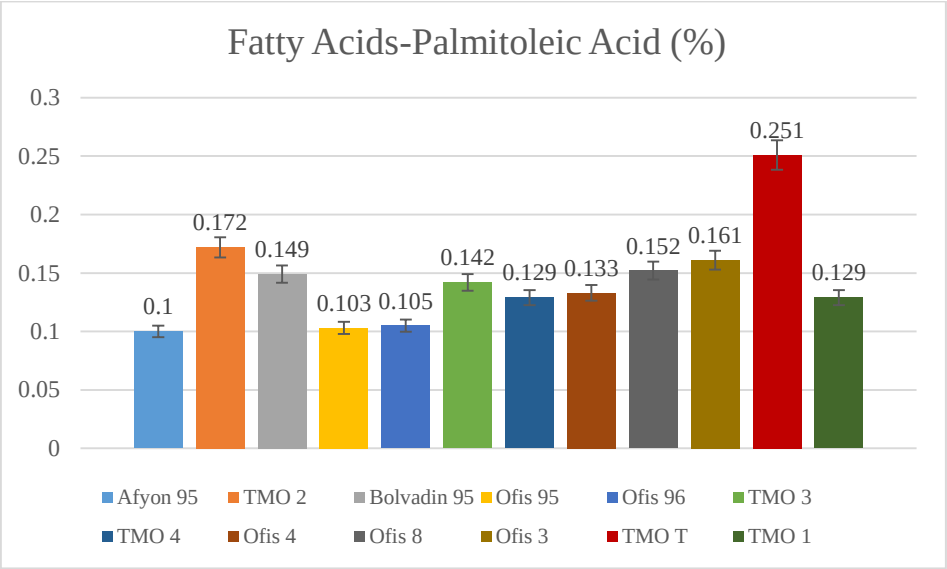

Figure 3.6 Percentage of palmitoleic acid in measured seeds of different *P. somniferum* cultivars

Palmitoleic acid rates can be listed descendingly as follows ; TMO T, Office 3, Office 8, TMO 2, Bolvadin 95, TMO 3, Office 4, TMO 4, TMO 1, Office 96, Office 95 and Afyon 95. Bozan and Temelli (2008) determined palmitoleic acid content of some poppy seeds as 0.13%.

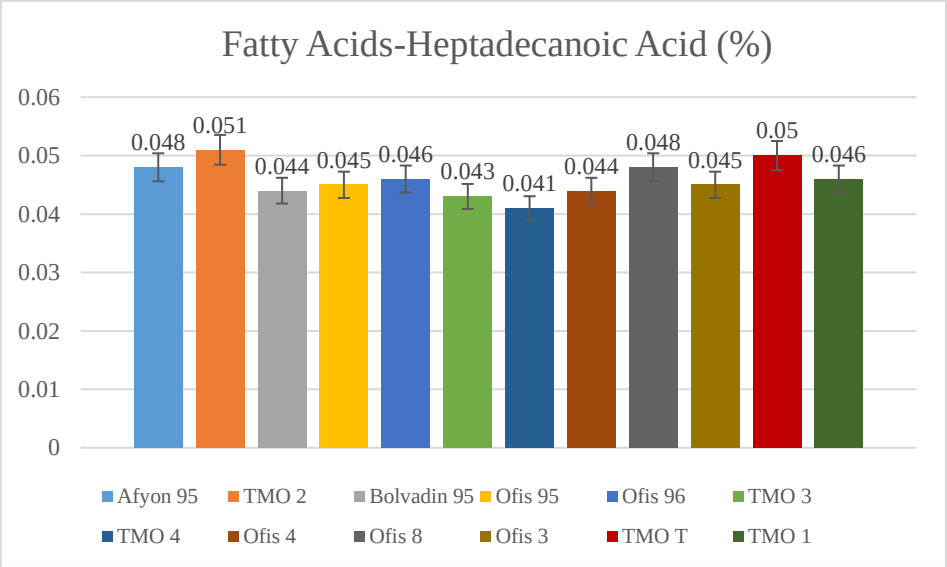

Figure 3.7 Percentage of heptadecanoic acid in measured seeds of different *P. somniferum* cultivars

Highest (0,051%) and lowest (0.041%) heptadecanoic acid rates were observed in TMO 2 and TMO 4 respectively while other cultivar have similar rates. Amir et al (2011) detected heptadecanoic acid percentage between 0.04-0.06% in poppy seeds which shows similar results to present study.

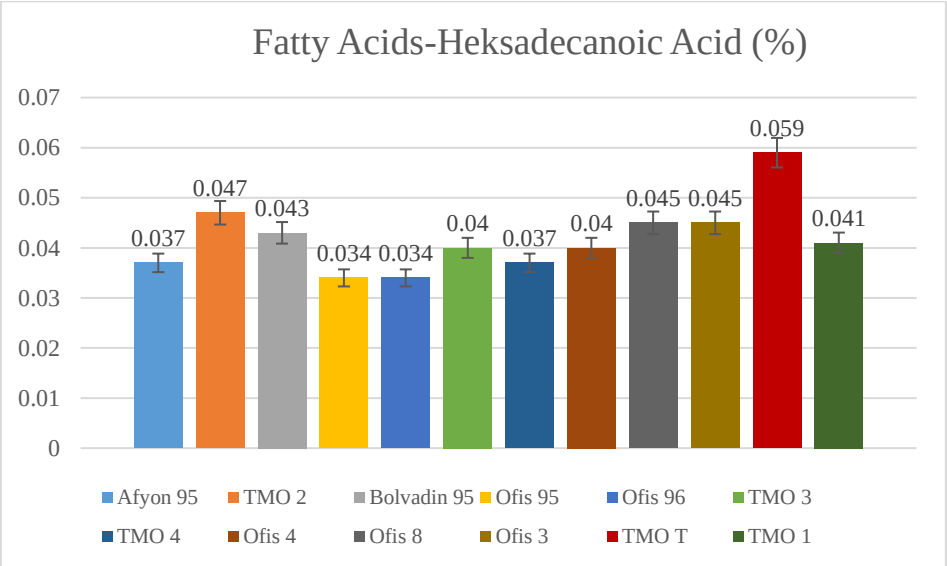

Figure 3.8 Percentage of heksadecanoic acid in measured seeds of different *P. somniferum* cultivars

The highest percentage of hexadecanoic acid is in the TMO T cultivar is lowest in the Ofis 95 and Ofis 96 cultivars.

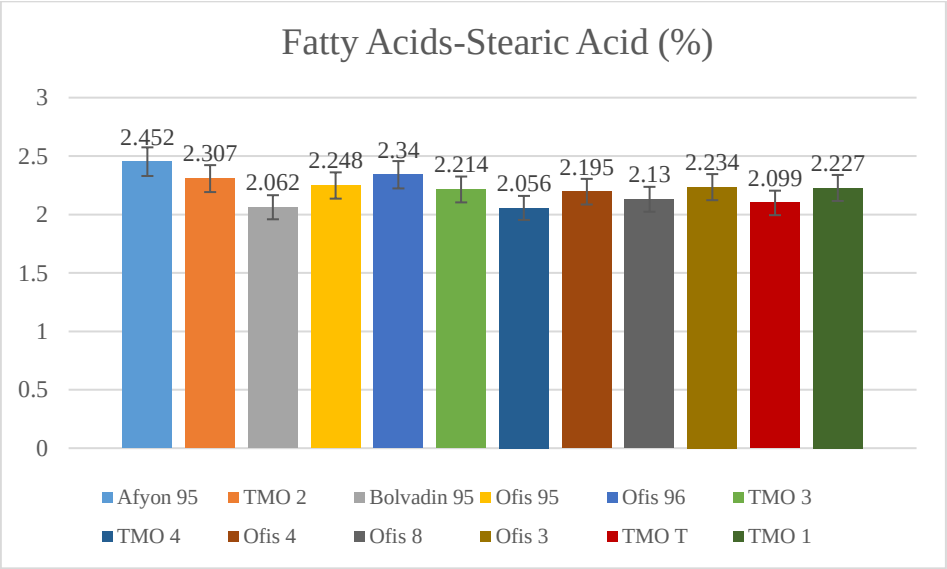

Figure 3.9 Percentage of stearic acid in measured seeds of different *P. somniferum* cultivars

It have been found that the cultivars have similar stearic acid percentages. It can be arranged from highest to lowest: Afyon 95, Ofis 96, TMO 2, Ofis 95, Ofis 3, TMO 1, TMO 3, Ofis 4, Ofis 8, TMO T, Bolvadin 95, TMO 4. Bozan and Temelli (2008) and Rahimi et al. (2011) analyzed the stearic acid content in opium poppy seeds and found similar results to this study while Ozcan and Atalay (2006) found stearic content as 2.40-4.30%, which is higher than this study.

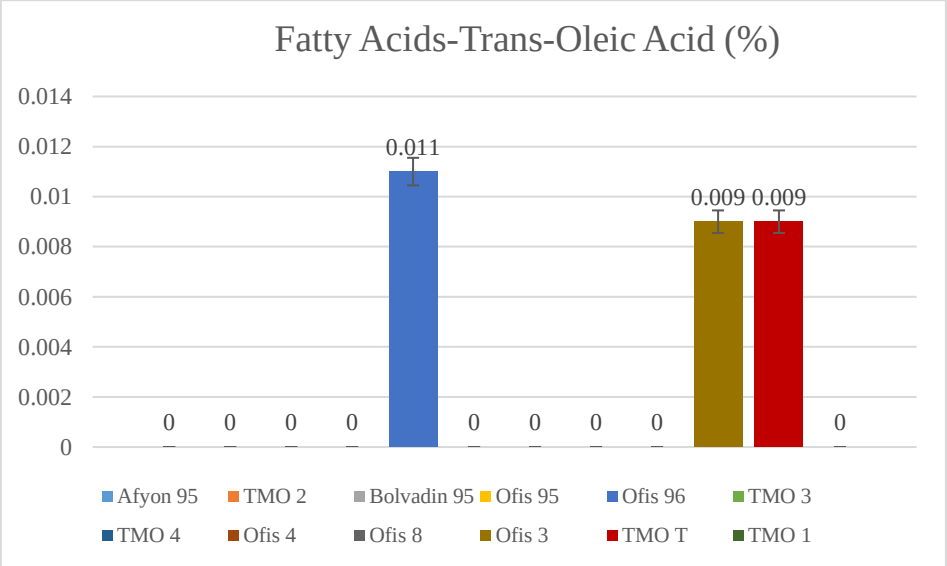

Figure 3.10 Percentage of trans-oleic acid in measured seeds of different *P. somniferum* cultivars

Trans-Oleic acid percentage were below the measurable value of many cultivars and were found to be 0.011% in the Ofis 96 cultivar and 0.009% in Ofis 3 and TMO T cultivars.

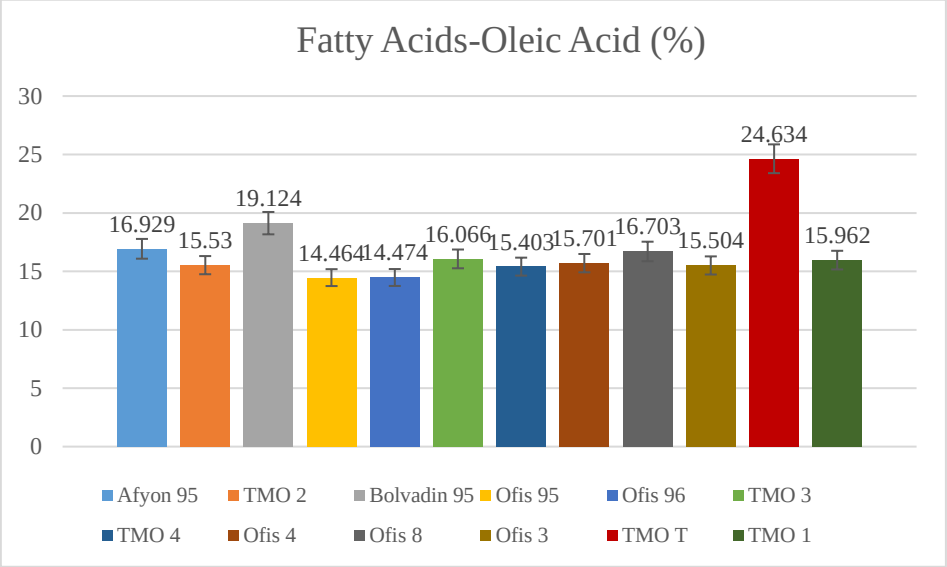

Figure 3.11 Percentage of oleic acid in measured seeds of different *P. somniferum* cultivars

According to results, highest level of oleic acid that is the second major fatty acid was found in cultivar TMO T. The others are decreasing as follows; Bolvadin 95, Afyon 95, Ofis 8, TMO 3, TMO 1, Ofis 4, TMO 2, Ofis 3, TMO 4, Ofis 96 and Ofis 95.

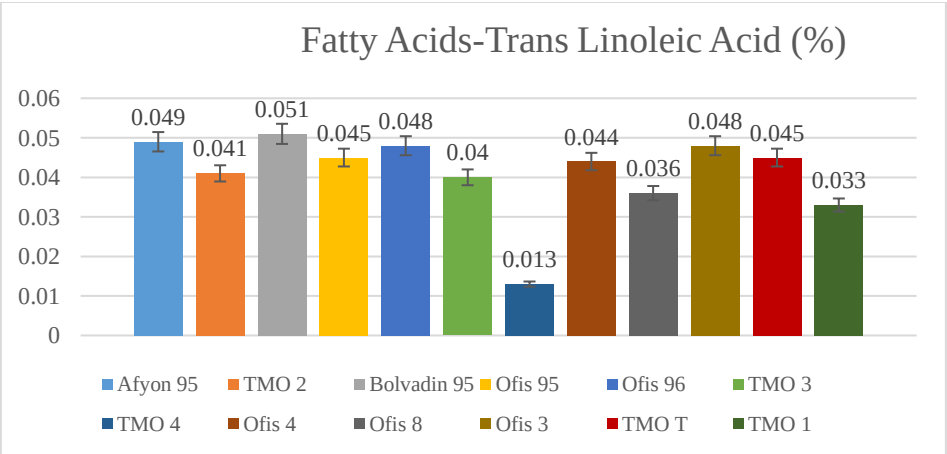

Figure 3.12 Percentage of trans-linoleic acid in measured seeds of different *P. somniferum* cultivars

When we analyzed Trans-Linoleic acid percentage, it was seen that Bolvadin 95 had the highest percentage and TMO 4 had the lowest percentage of cultivars.

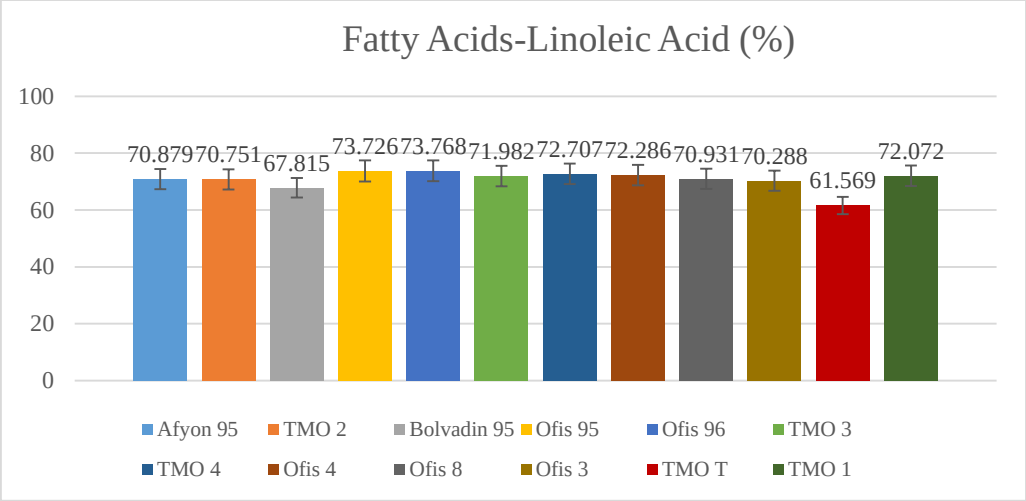

Figure 3.13 Percentage of linoleic acid in measured seeds of different *P.somniferum* cultivars

Linoleic acid is the most abundant fatty acid in the seeds of all cultivars. When comparing cultivars, Ofis 96 cultivar has the largest percentage and TMO T variety has the lowest percentage.

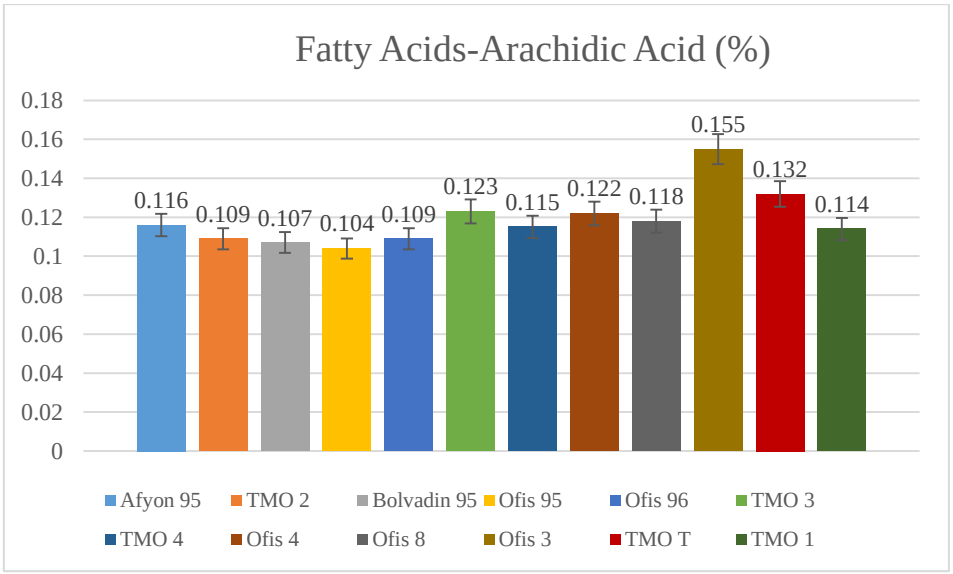

Figure 3.14 Percentage of arachidic acid in measured seeds of different *P. somniferum* cultivars

Small amounts of arachidic acid values were measured in the seeds of the *P. somniferum* cultivars, with a maximum of one hundred percent cutivars, with 0,155% in Office 3.

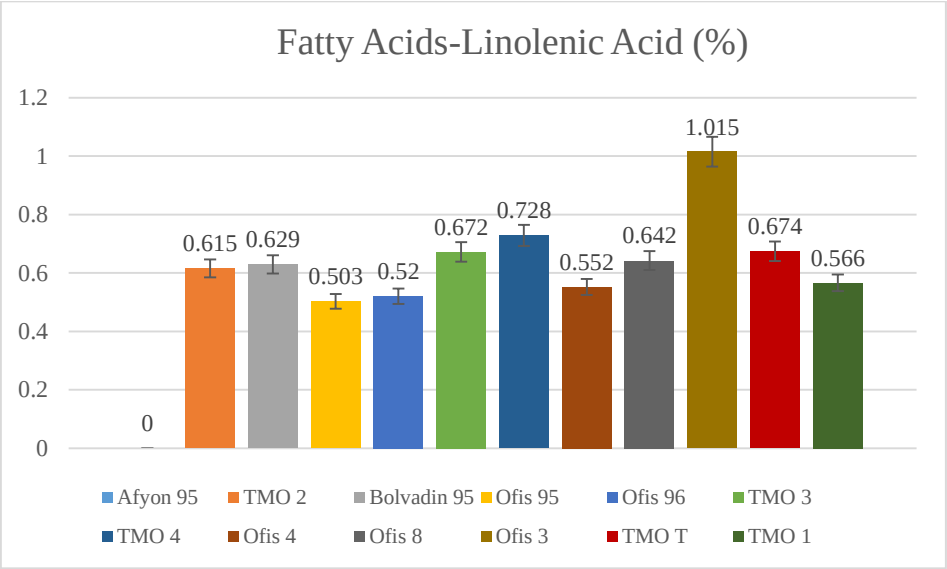

Figure 3.15 Percentage of linolenic acid in measured seeds of different *P.somniferum* cultivars

Opium seed's linolenic acid value was detected below the limit value that can be measured in Afyon 95 cultivar. Compared correct ranking from high to low values in other cultivars are as follows: Ofis 3, TMO 4, TMO T, TMO 3, Ofis 8, Bolvadin 95, TMO 2, TMO 1, Ofis 4, Ofis 96 and Ofis 95.

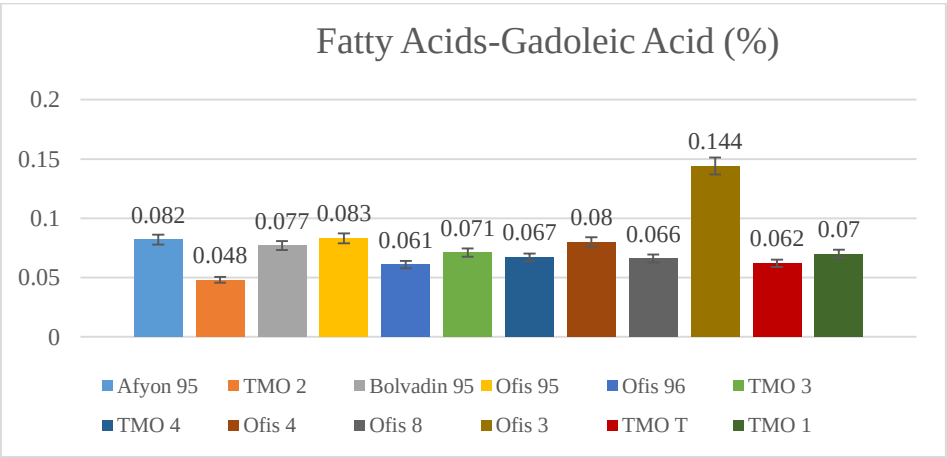

Figure 3.16 Percentage of gadoleic acid in measured seeds of different *P. somniferum* cultivars

It has been determined that the gadoleic acid value of the seeds of the Ofis 3 cultivar is about twice that of the seeds of the other cultivars.

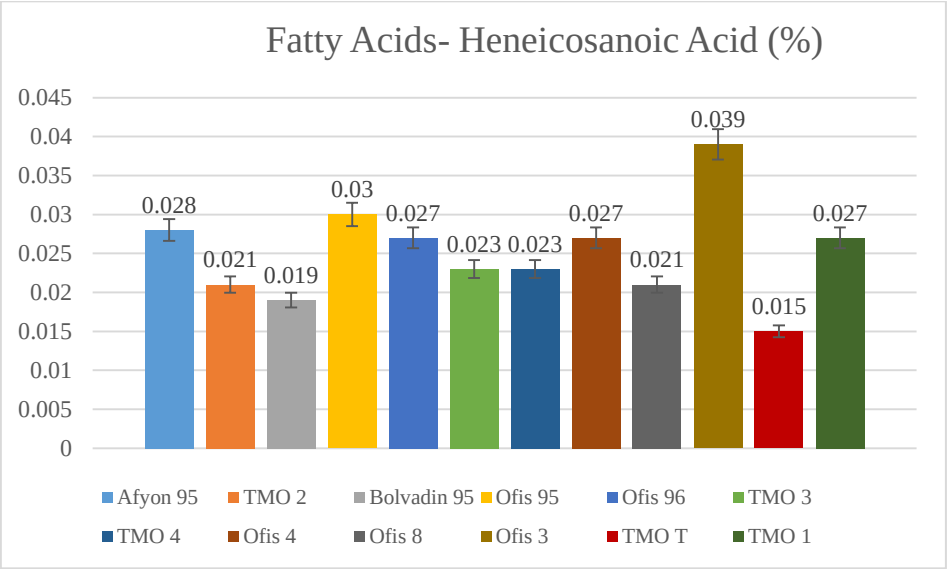

Figure 3.17 Percentage of heneicosanoic acid in measured seeds of different *P.somniferum* cultivars

Heneicosanoic acid percentages can be ordered from the highest to the lowest;Ofis 3, Ofis 95, Afyon 95, Ofis 96, Ofis 4, TMO 1, TMO 3, TMO 4, TMO 2, Ofis 8, Bolvadin 95 and TMO T.

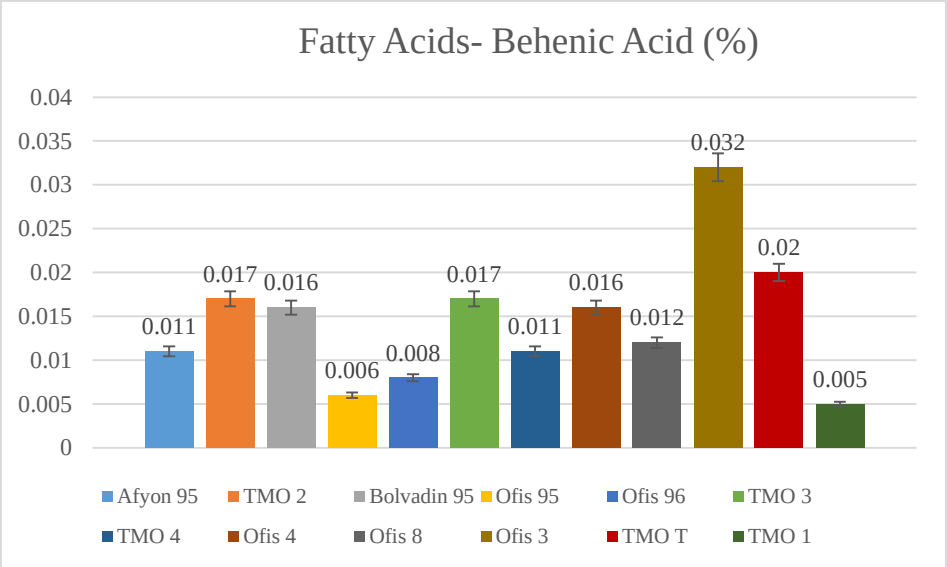

Figure 3.18 Percentage of behenic acid in measured seeds of different *P.somniferum* cultivars

Ofis 3 has the highest and TMO 1 has the lowest percentage of behenic acid.

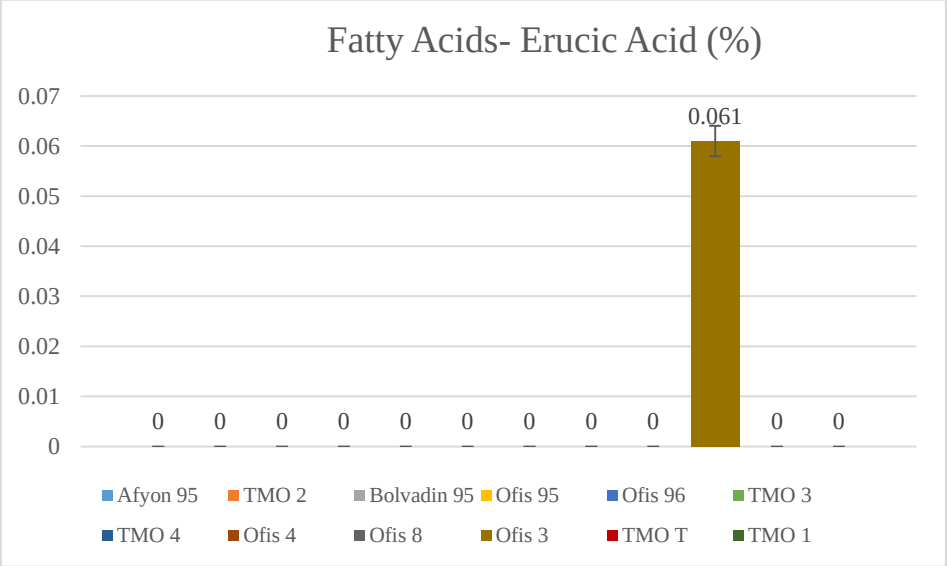

Figure 3.19 Percentage of erucic acid in measured seeds of different *P.somniferum* cultivars

Only Office 3 cultivar were found to be above the measurable values of erucic acid.

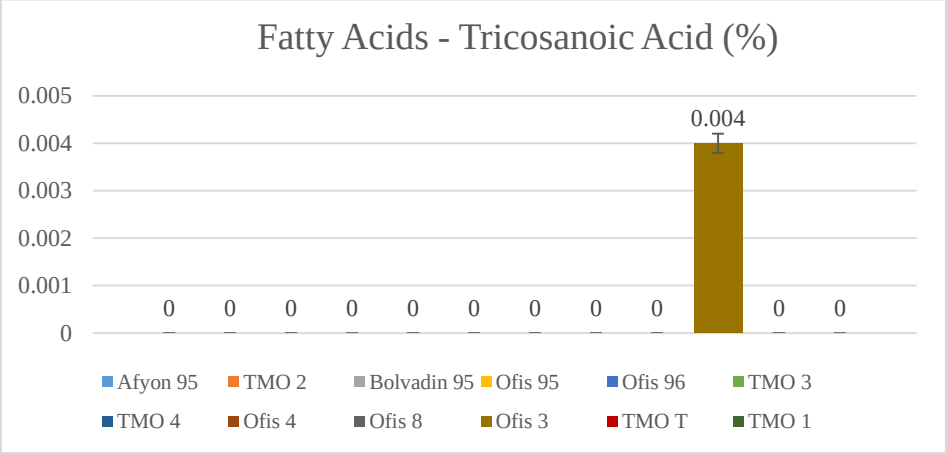

Figure 3.20 Percentage of tricosanoic acid in measured seeds of different *P. somniferum* cultivars

Only Office 3 cultivar were found to be above the measurable values of tricosanoic acid.

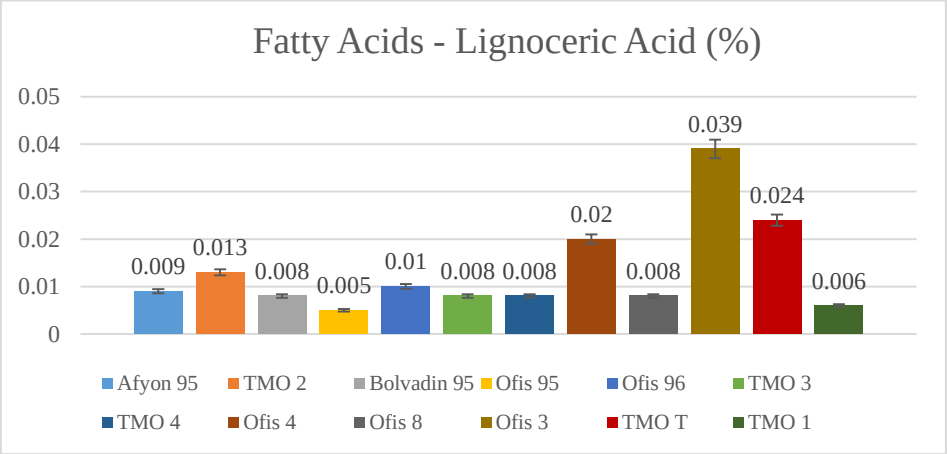

Figure 3.21 Percentage of lignoceric acid in measured seeds of different *P.somniferum* cultivars

Lignoceric acid percentages are as follows descending; Ofis 3, TMO T, TMO 2, Ofis 96, Afyon 95, Bolvadin 95, TMO 3, TMO 4, OFis 8, TMO 1, Ofis 95,

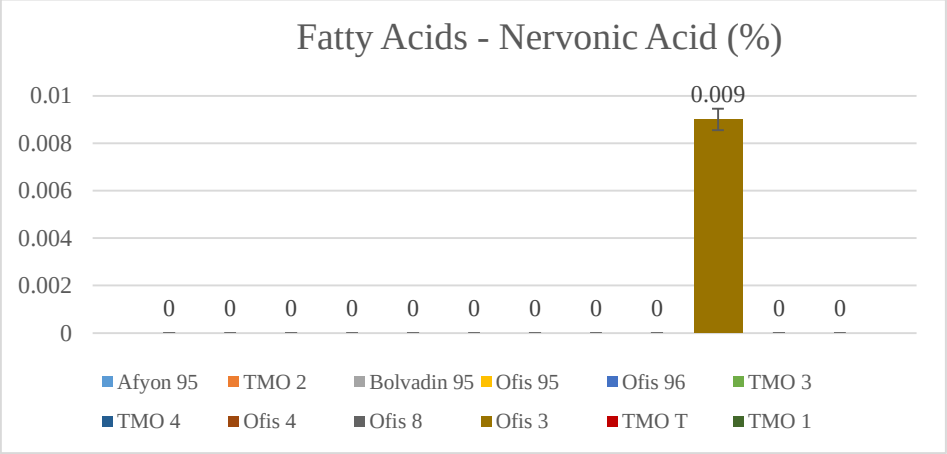

Figure 3.22 Percentage of nervonic acid in measured seeds of different *P.somniferum* cultivars

Only Office 3 cultivar were found to be above the measurable values of nervonic acid.

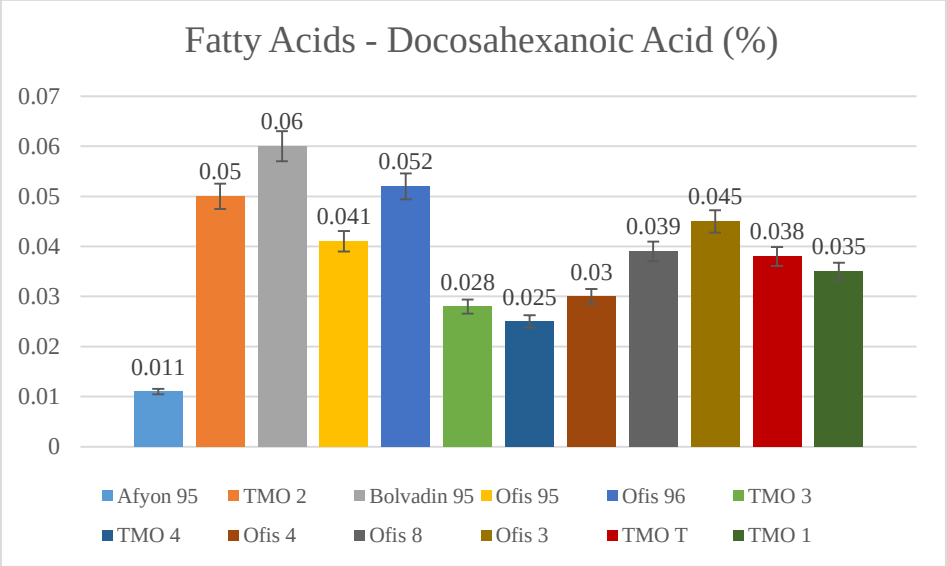

Figure 3.23 Percentage of docosahexanoic acid in measured seeds of different *P. somniferum* cultivars

Bolvadin 95 has the highest and Afyon 95 has the lowest docosahexanoic acid percentage.

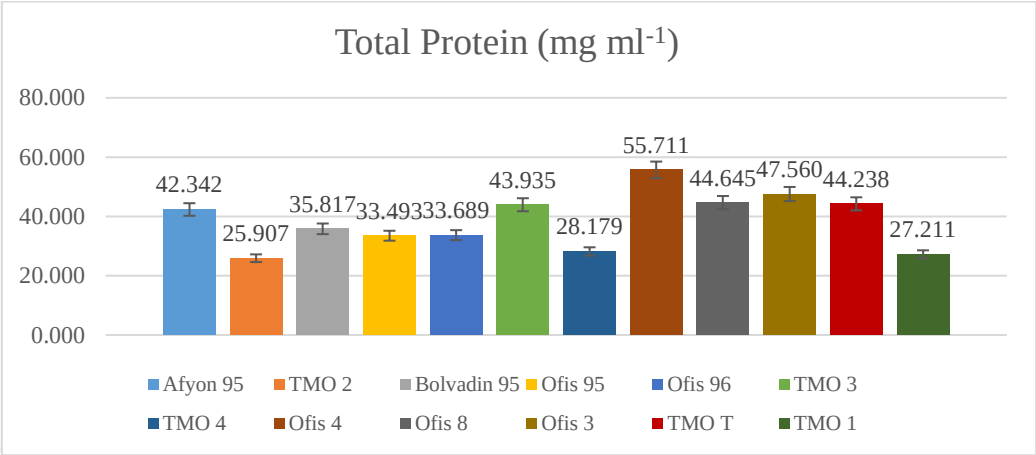

Figure 3.24 Amount of total protein in seed of different *P.somniferum* cultivars

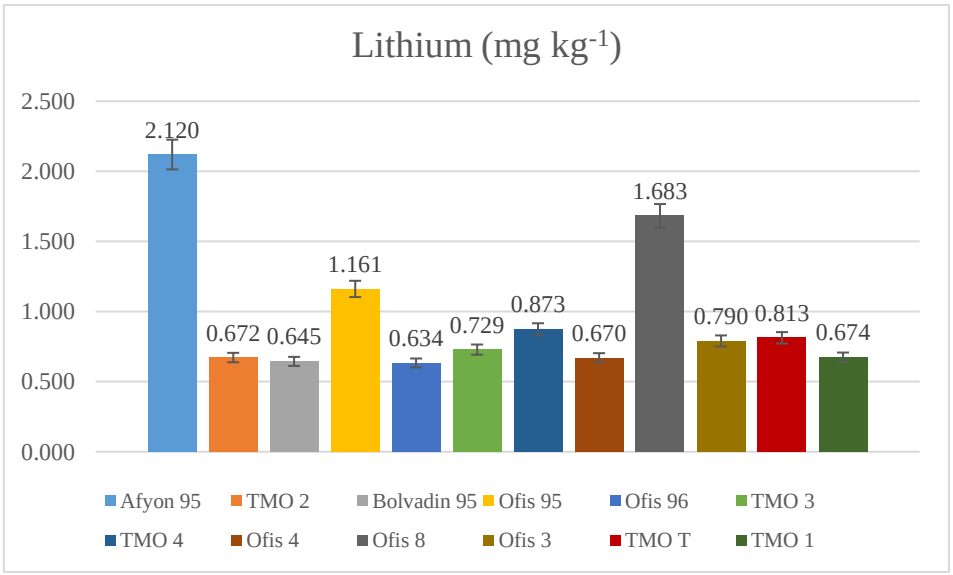

Figure 3.25 Lithium amount in measured seeds of different *P. somniferum* cultivars

Lithium concentration was found highest in Afyon 95 cultivar and the lowest in Ofis 96. From the highest to the lowest all used cultivars are as follows: Ofis 8, Ofis 95 , TMO 4, TMO T, Ofis 3, TMO 3, TMO 1, TMO 2, Ofis 4, Bolvadin 95. Ozcan (2004) reported lithium amount for opium poppy seed as 0,09 mg kg^{-1}.

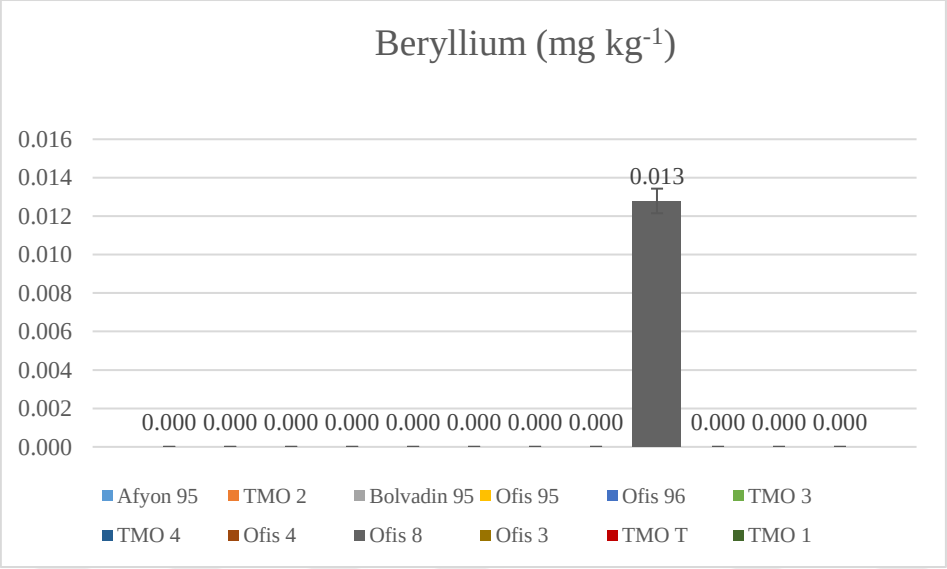

Figure 3.26 Beryllium amount in measured seeds of different *P. somniferum* cultivars

Beryllium was measured as 0,013 mg kg^{-1} only in of the Ofis 8 cultivar.

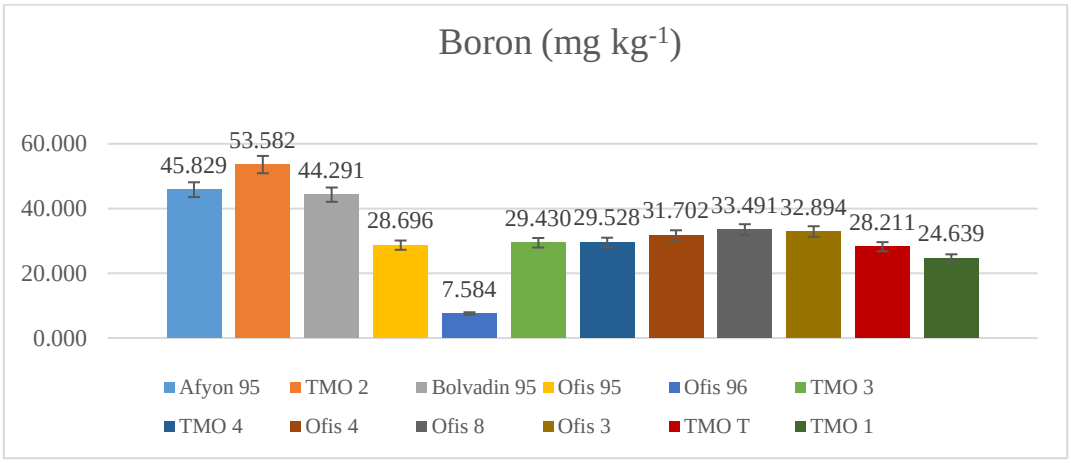

Figure 3.27 Boron amount in measured seeds of different *P. somniferum* cultivars

When boron values are compared, TMO 2 has the most boron content whereas Ofis 96 has the least with a significant difference. Afyon 95, Bolvadin 95 and TMO 2 boron values are relatively high according to other varieties.

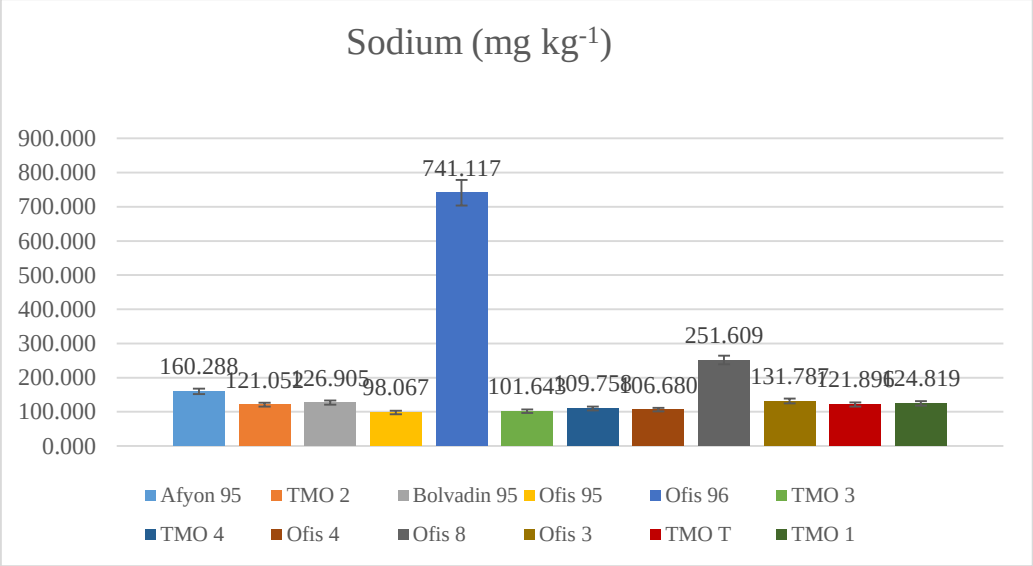

Figure 3.28 Sodium amount in measured seeds of different *P. somniferum* cultivars

Na contents of varieties are appeared to be at the same line except Afyon 95, Ofis 8 and Ofis 96. Na amount in Ofis 8 is distinctively high while Afyon 95 has almost similar to others. Additionally, Ofis 96 has the lowest value.

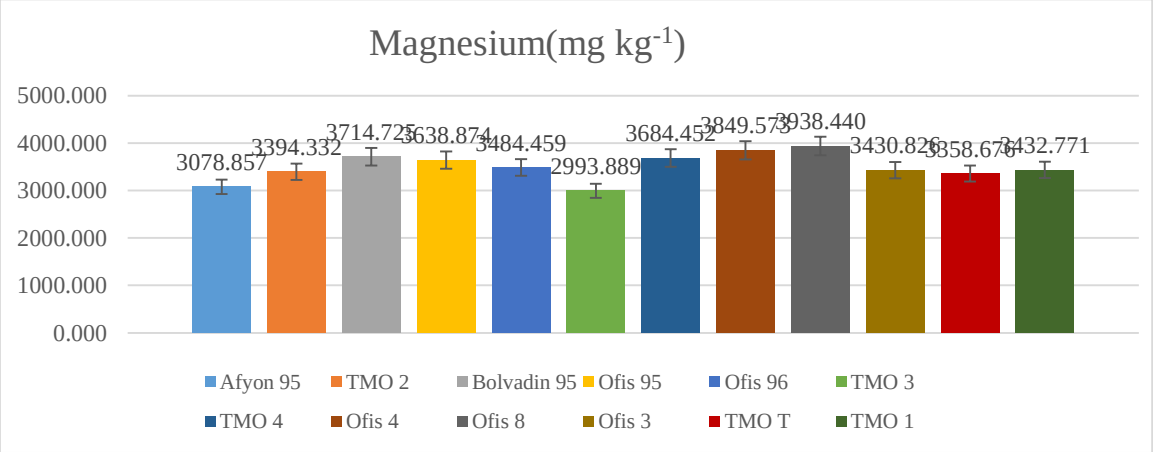

Figure 3.29 Magnesium amount in measured seeds of different *P.somniferum* cultivars

There is no significant difference between Mg levels of seeds except Ofis 95. Mg content of Ofis 95 is relatively low compared to other cultivars. Ozcan (2006) also found similar Mg amounts in different Turkish opium poppy seeds.

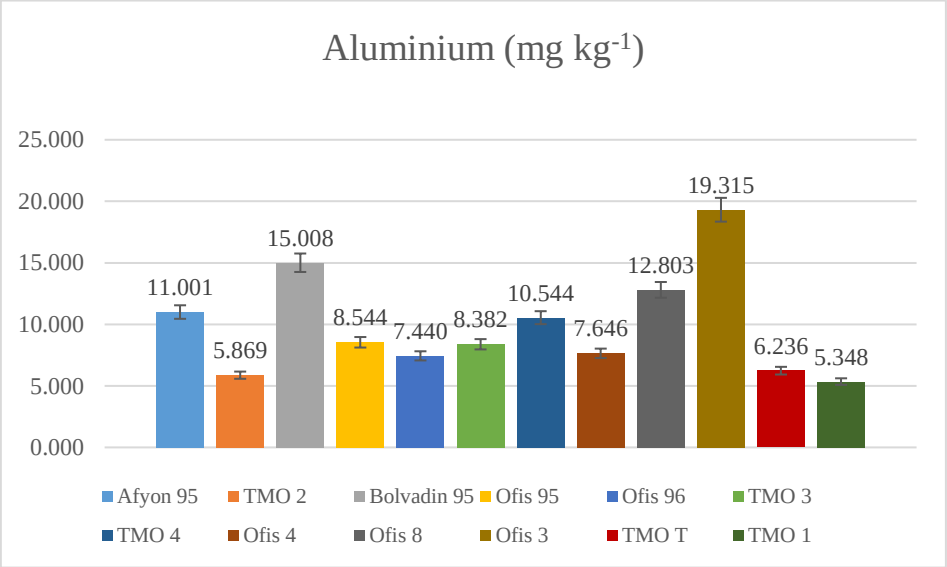

Figure 3.30 Aluminum amount in measured seeds of different *P. somniferum* cultivars

Aluminum contents of cultivars are distinctively different and Ofis 3 (19.315 mg/kg) has the highest value while Ofis 96 (2.939 mg/kg) has the lowest.

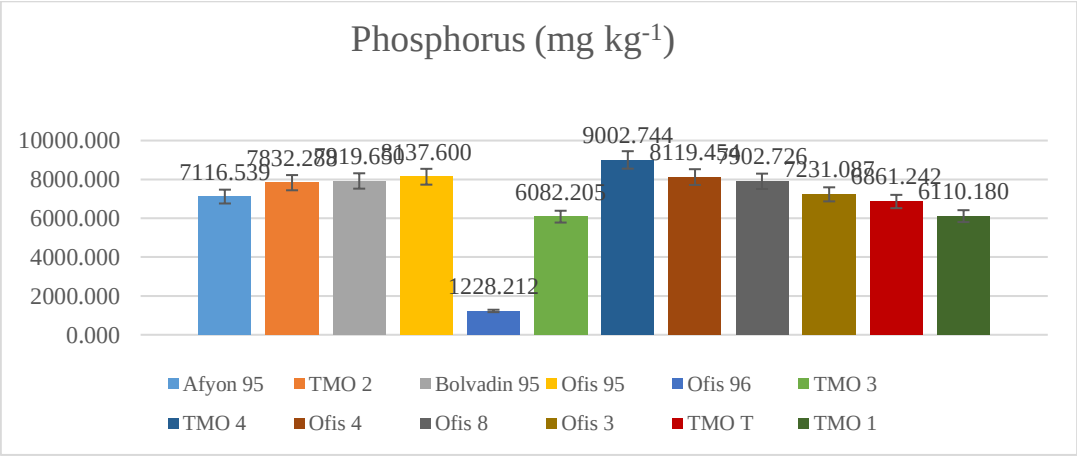

Figure 3.31 Phosphorus amount in measured seeds of different *P. somniferum* cultivars

Most of studied opium poppy seeds have relatively high phosphorus content apart from Ofis 96, TMO 3 and TMO 1 as these are significantly low compared to others. Ozcan (2006) found phosphorus levels of different opium poppy cultivar seeds at similar levels in agreement with this study.

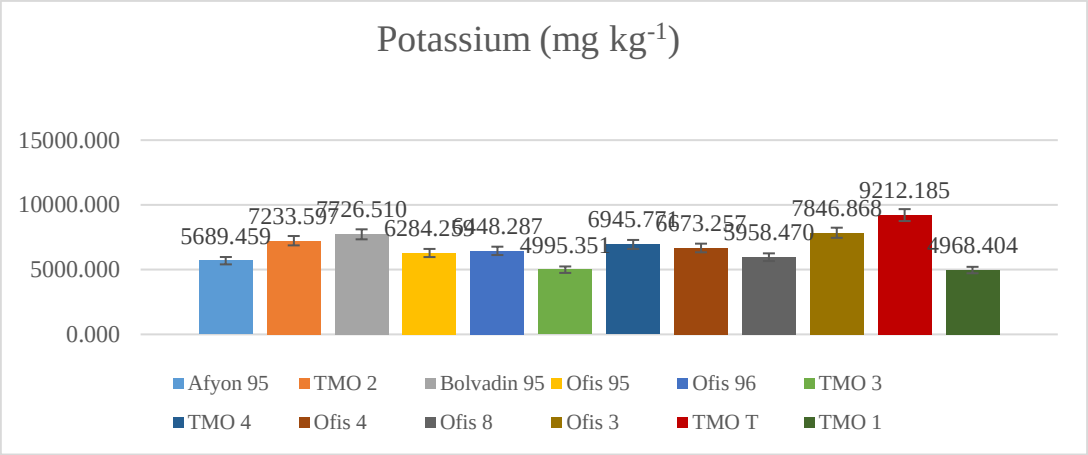

Figure 3.32 Potassium amount in measured seeds of different *P.somniferum* cultivars

Potassium levels vary by cultivar. K contents of seeds are as follows from highest to lowest: TMO T (9212.185 mg/kg), Ofis 3 (7846. 868 mg/kg), Bolvadin 95 (7726.510 mg/kg), TMO 2 (7233.597 mg/kg), TMO 4 (6945.771 mg/kg), Ofis 4 (6673.257 mg/kg), Ofis 96 (6448.287 mg/kg), Ofis 95 (6284.255 mg/kg), Ofis 8 (5958.470 mg/kg), Afyon 95 (5689.459 mg/kg), TMO 3 (4995.351 mg/kg), TMO 1 (4968.404 mg/kg).

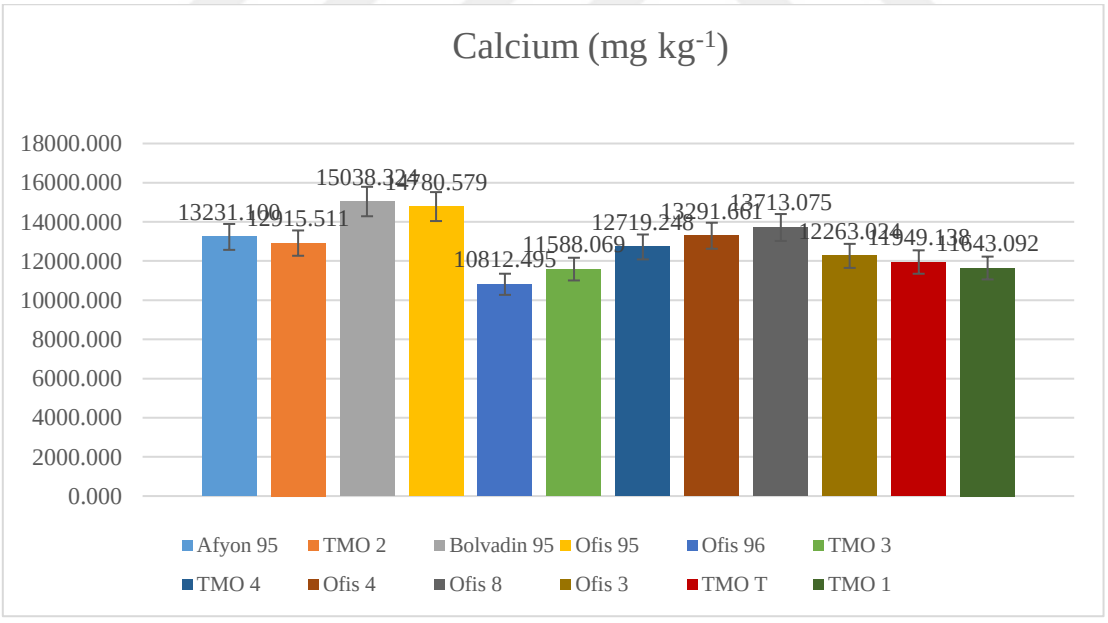

Figure 3.33 Calcium amount in measured seeds of different *P. somniferum* cultivars

It is observed that there is not a remarkable difference of Ca content between different cultivars as concentrations ranges from 10.812 to15.038 mg/kg. Nevertheless, Bolvadin 95 and Ofis 95 seeds have relatively high Ca content compared to others.

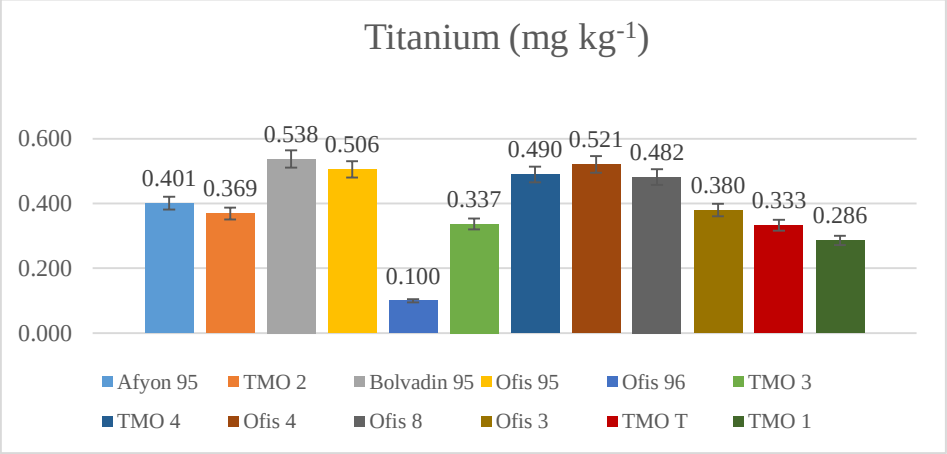

Figure 3.34 Titanium amount in measured seeds of different *P.somniferum* cultivars

It is observed that Ti amounts of seeds are highly variable. Bolvadin 95 has the highest Ti content (0.538 mg/kg) whereas Ofis 96 has the lowest (0.100 mg/kg).

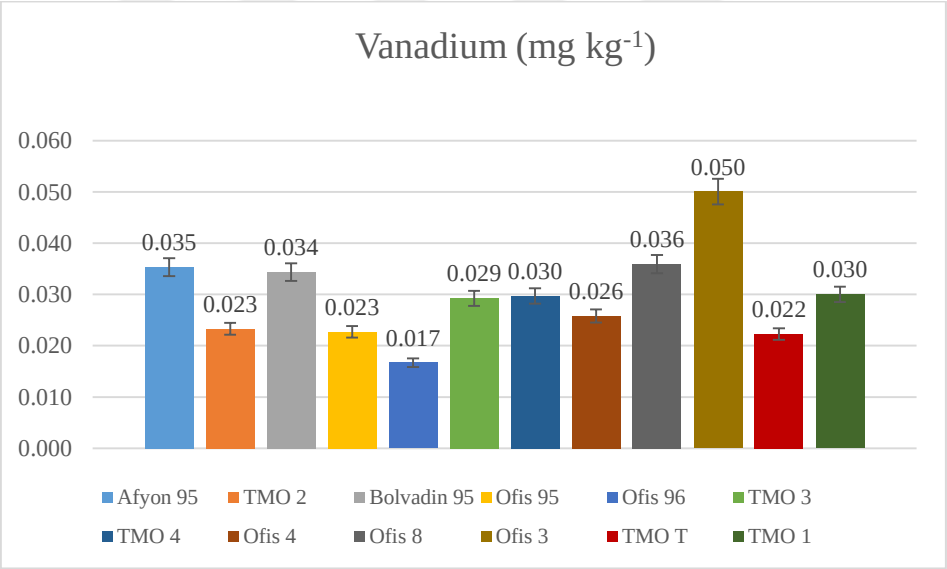

Figure 3.35 Vanadium amount in measured seeds of different *P. somniferum* cultivars

Antal et al. (2009) reported V content of some medicinal plants and found 0.502 mg/kg of V content in dry weight. As it is shown in Figure 3.35, V contents are vary by cultivar between 0.017 and 0.050 mg/kg.

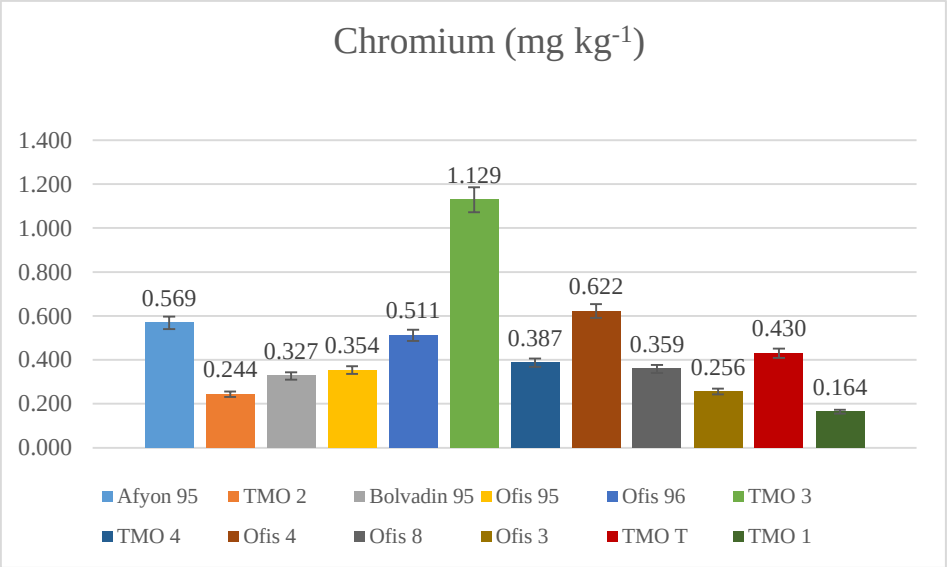

Figure 3.36 Chromium amount in measured seeds of different *P. somniferum* cultivars

Chromium contents of seeds are also fluctuates between 0.164 mg/kg (TMO 1) and 1.129 mg/kg (TMO 3). Additionally, Cr amount of TMO 3 seed significantly high (0.164 mg/kg) compared to others.

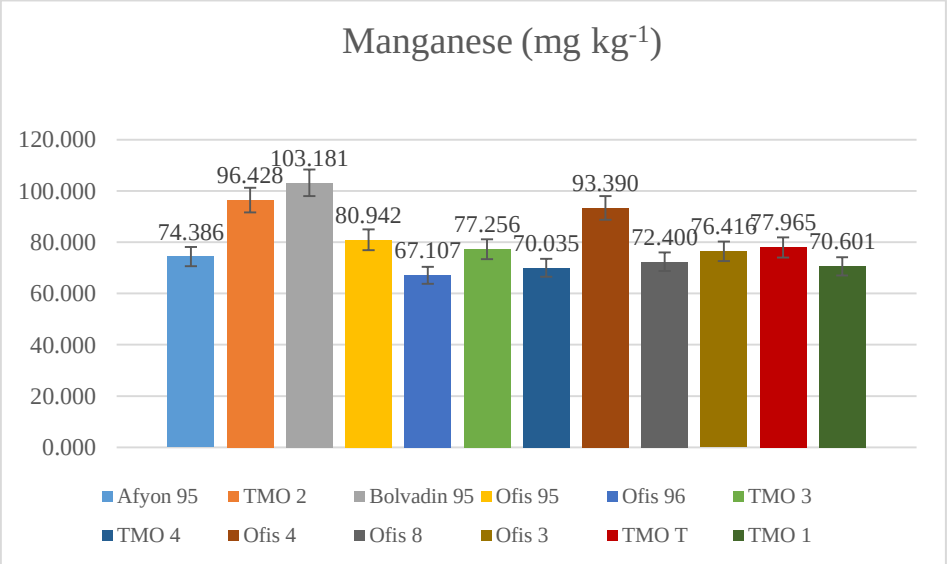

Figure 3.37 Manganese amount in measured seeds of different *P. somniferum* cultivars

Varying levels are observed in Mn concentrations. Mn contents of all cultivars are as follows from highest to lowest: 103.181 mg/kg (Bolvadin 95), 96.428 mg/kg (TMO 2), 93.390 mg/kg (Ofis 4), 80.942 mg/kg (Ofis 95), 77.965 mg/kg (TMO T), 77.256 mg/kg (TMO 3), 76.416 mg/kg (Ofis 3), 74.386 mg/kg (Afyon 95), 72.400 (Ofis 8), 70.601 mg/kg (TMO 1), 70.035 mg/kg (TMO 4) and 67.107 mg/kg (Ofis 96).

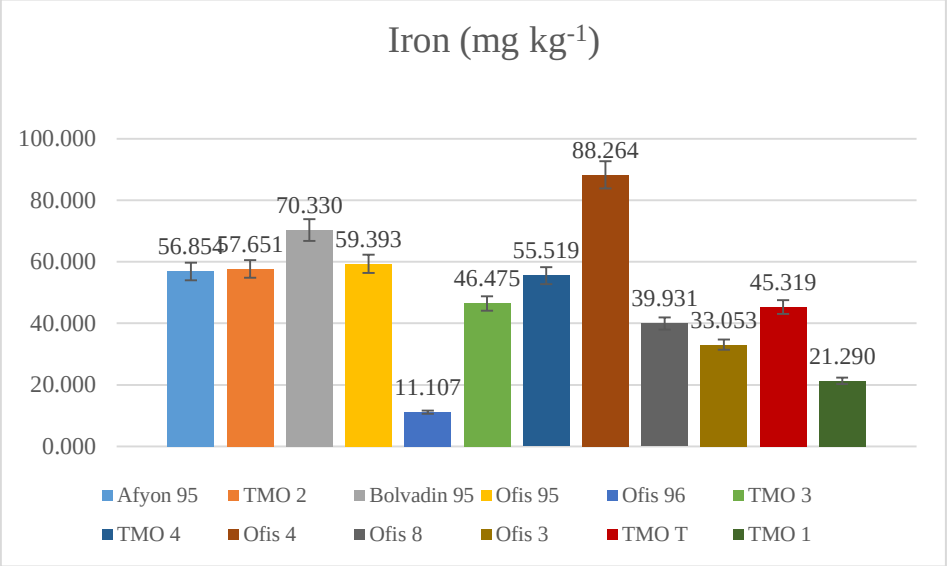

Figure 3.38 Iron amount in measured seeds of different *P. somniferum* cultivars

Fe contents of Afyon 95 (56.854 mg/kg), TMO 2 (57.651 mg/kg), Ofis 95 (59.393 mg/kg) and TMO 4 (55.519 mg/kg) are close at medium levels while Ofis 3 has the highest Fe (88.264 mg/kg) content and Ofis 96 has the lowest (11.107 mg/kg). Ozcan and Atalay (2006) observed Fe contents of different opium poppy seeds between 64.1 and 104.5 mg/kg.

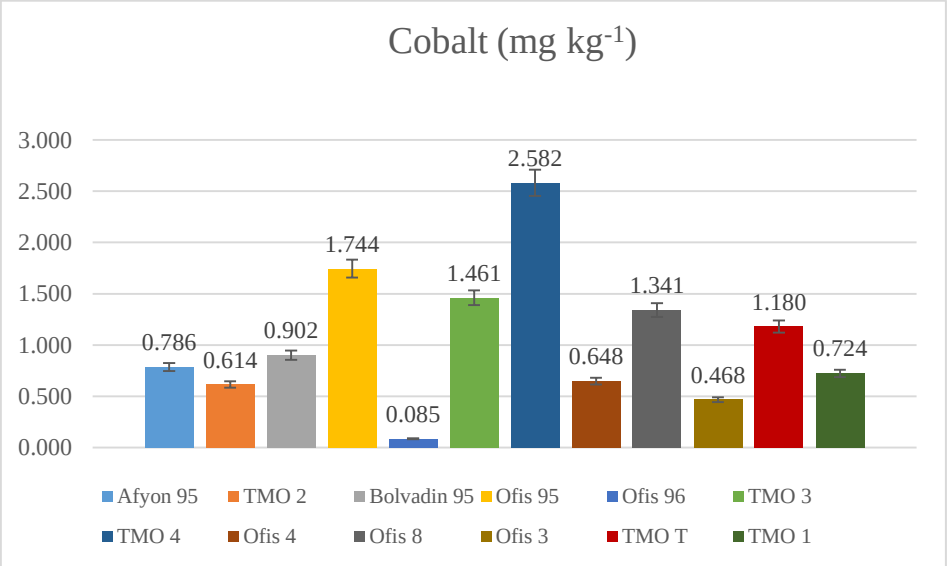

Figure 3.39 Cobalt amount in measured seeds of different *P. somniferum* cultivars

Co levels of Ofis 95 (1.744 mg/kg), TMO 3 (1.461 mg/kg) and Ofis 8 (1.341 mg/kg) are similar at average level while TMO 4 has the highest Co content (2.582 mg/kg) and Ofis 96 has the lowest (0.085 mg/kg).

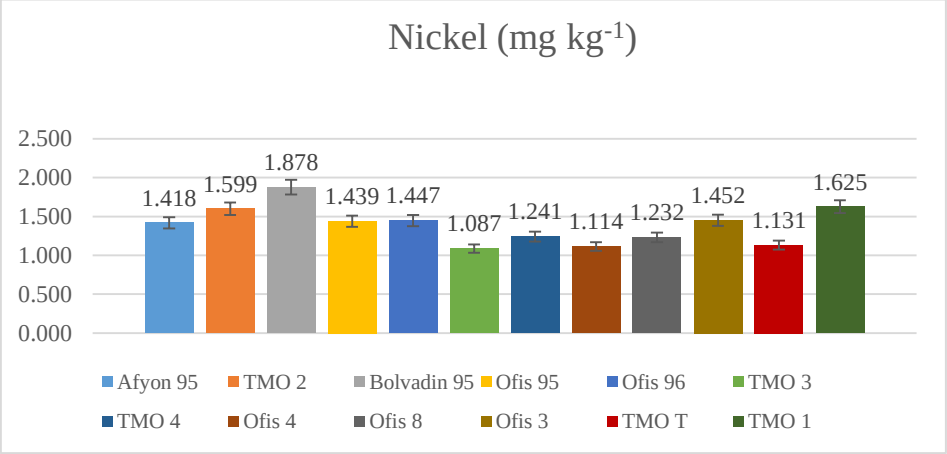

Figure 3.40 Nickel amount in measured seeds of different *P. somniferum* cultivars

It is determined that Ni contents of all seeds show similar levels range between 1.087 (TMO 3) and 1.878 mg/kg (Bolvadin 95).

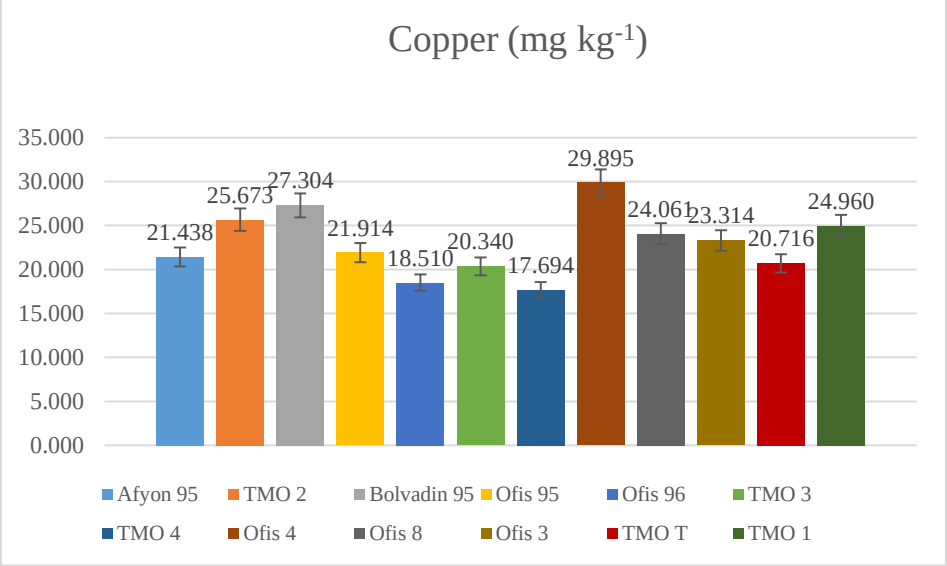

Figure 3.41 Copper amount in measured seeds of different *P. somniferum* cultivars

Cu amounts ranges from 17.964 (TMO 4) to 29.895 mg/kg (Ofis 4) while TMO 1, Ofis 8 and TMO 2 levels are at mid-range.

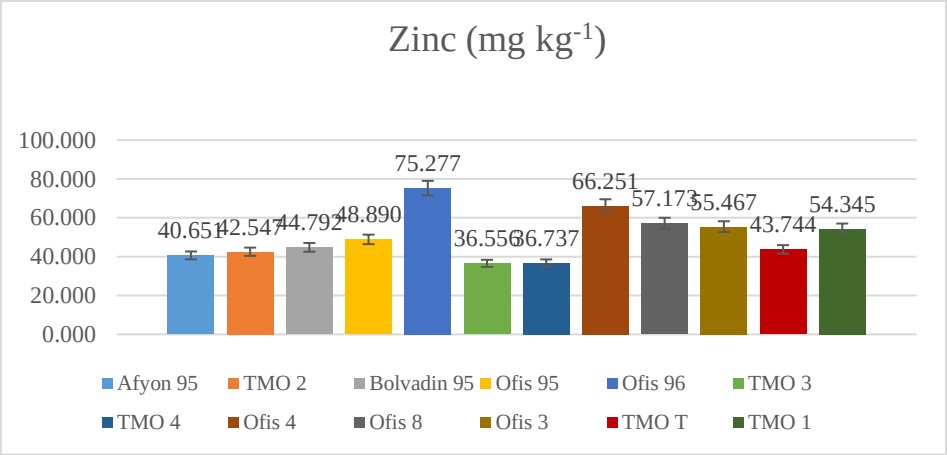

Figure 3.42 Zinc amount in measured seeds of different *P. somniferum* cultivars

Some cultivars are appear to have same level of Zn concentrations. Ofis 8, Ofis 3 and TMO 1 contain approximately same Zn content at mid-range. There is significant difference between the highest (75.277 mg/kg) and the lowest (36.556 mg/kg) concentrations.

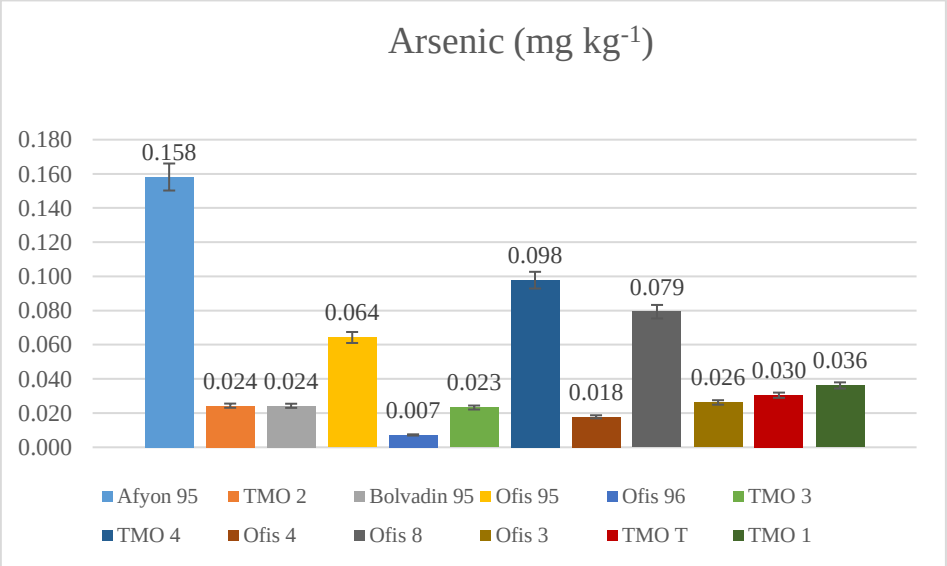

Figure 3.43 Arsenic amount in measured seeds of different *P. somniferum* cultivars

All seeds except Afyon 95, Ofis 95, TMO 4 and Ofis 8 show similar levels of as contents while others have significantly high level of concentrataions.

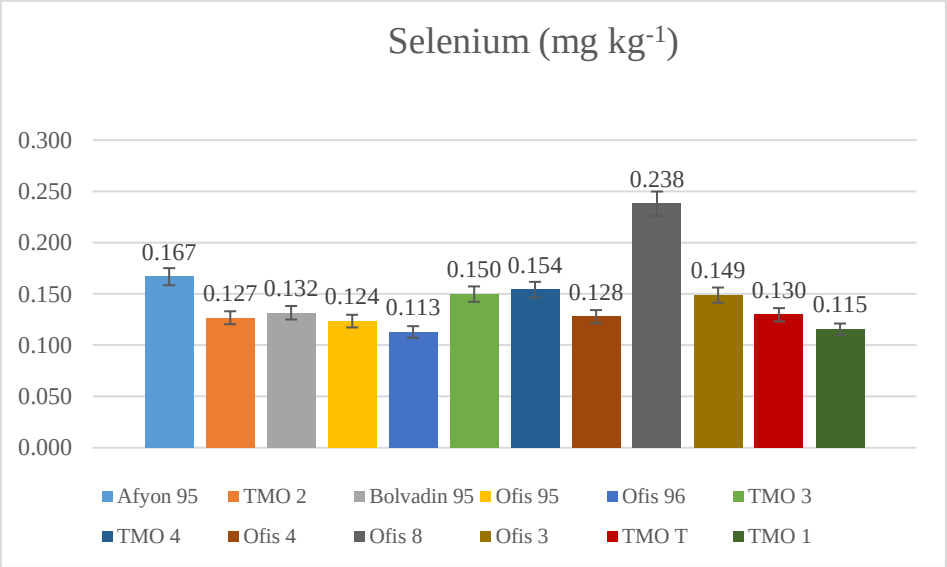

Figure 3.44 Selenium amount in measured seeds of different *P. somniferum* cultivars

Se levels of seeds ranges from 0.113 (Ofis 96) to 0.238 mg/kg (Ofis 8). TMO 3, TMO 4 and Ofis 3 shows similar level of concentrations close to mid-range.

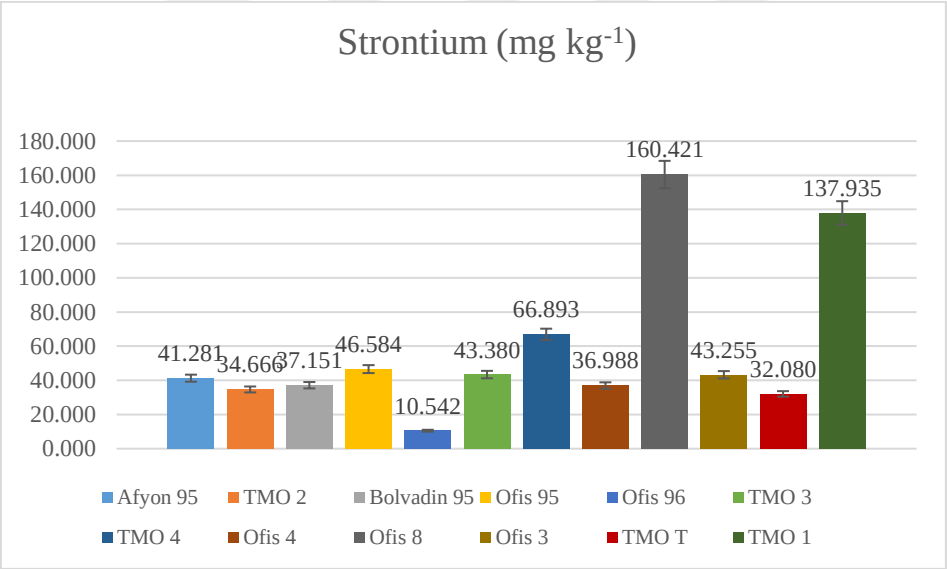

Figure 3.45 Strontium amount in measured seeds of different *P. somniferum* cultivars

Sr contents of plants distinctively vary by species (Kabata-Pendias, 2011). In agreement with that information, all cultivars have significantly different amounts of Sr ranges between 160.421 (Ofis 8) and 10.542 (Ofis 96).

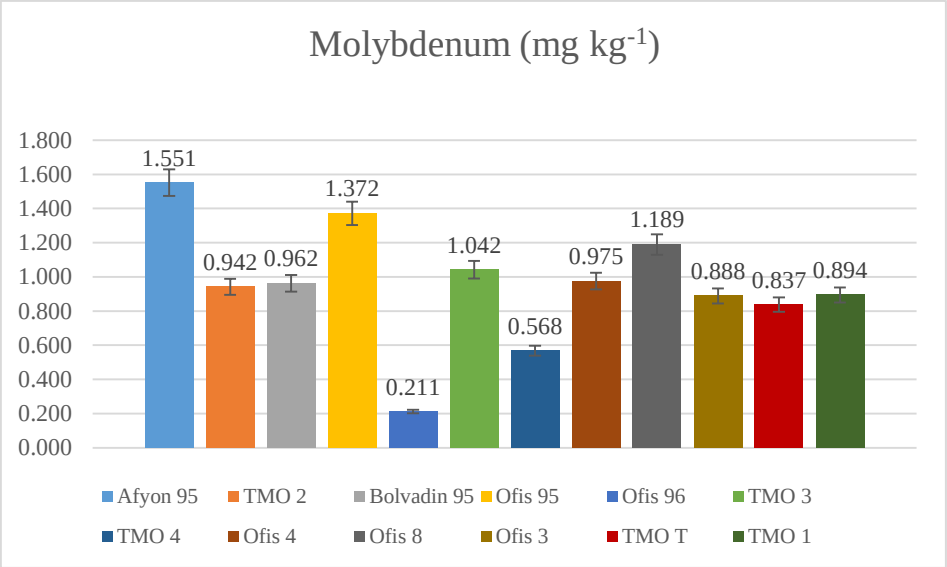

Figure 3.46 Molybdenum amount in measured seeds of different *P. somniferum* cultivars

Mo levels are also have a wide range from 0.211 (Ofis 96) to 1.551 mg/kg (Afyon 95) while Ofis 3, TMO T and TMO 1 have similar Mo contents close to mid-range.

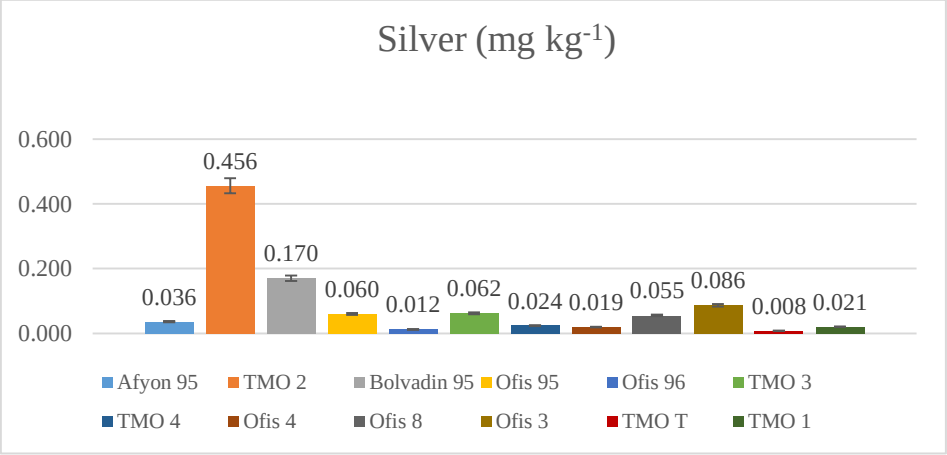

Figure 3.47 Silver amount in measured seeds of different *P. somniferum* cultivars

There is a wide range between the highest (0.456 mg/kg) and the lowest (0.008 mg/kg) Ag content. All levels are below mid-range except TMO 2 where other values are highly variable in a relatively small range.

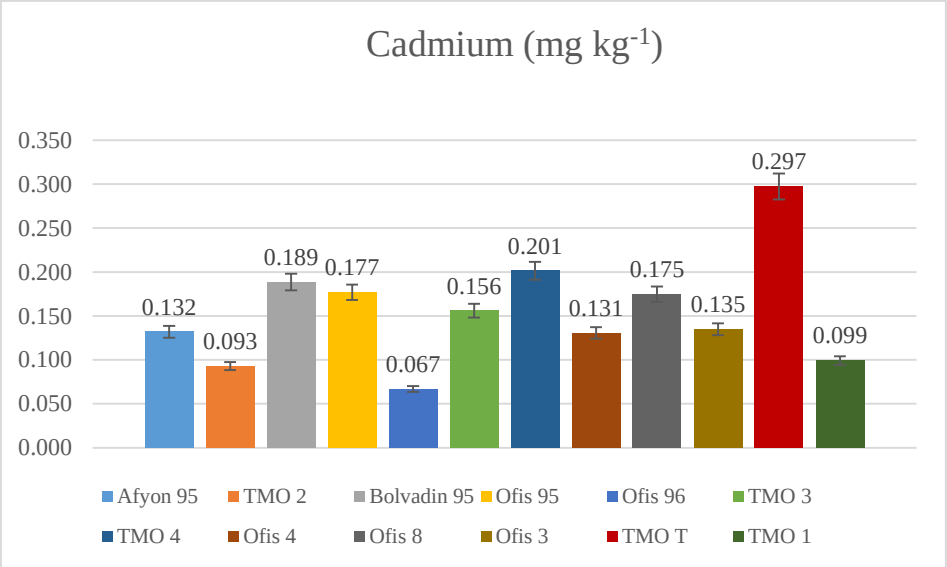

Figure 3.48 Cadmium amount in measured seeds of different *P. somniferum* cultivars

There is a fluctuation in Cd contents of seeds ranges from 0.067 (Ofis 96) to 0.297 mg/kg (TMO T) where Bolvadin 95 (0.189 mg/kg), Ofis 95 (0.177 mg/kg) and Ofis 8 (0.175 mg/kg) showed average levels compared to other values.

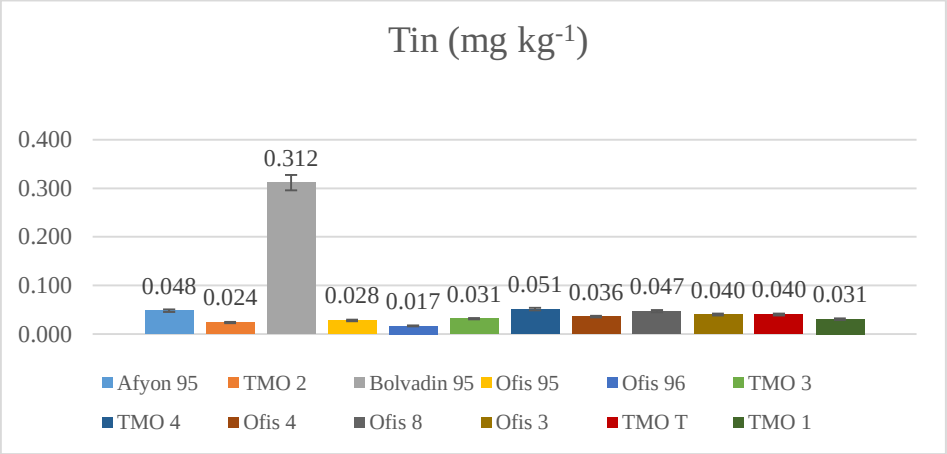

Figure 3.49 Tin amount in measured seeds of different *P. somniferum* cultivars

Sn concentrations of opium poppy seeds are appear to be at similar levels while Bolvadin 95 has distinctively high Sn content (0.312 mg/kg).

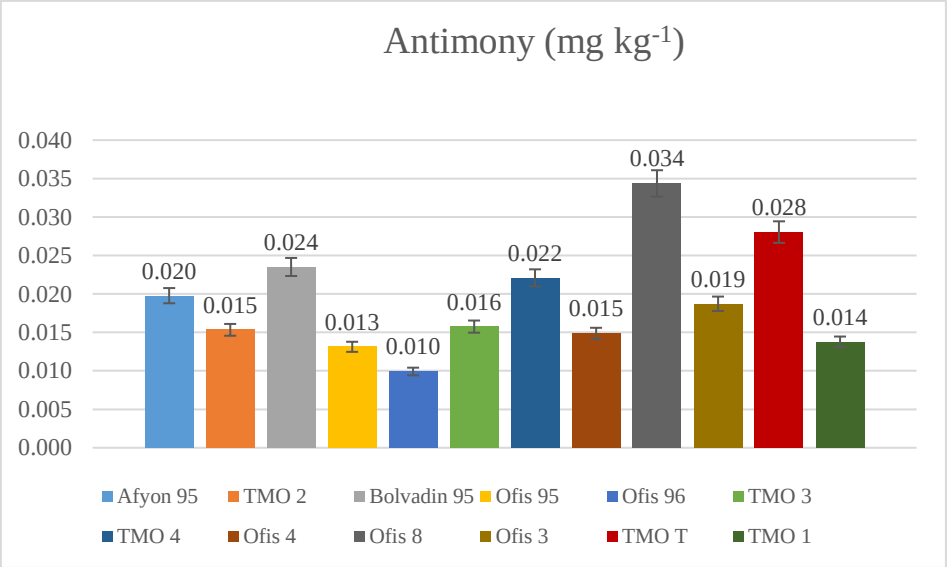

Figure 3.50 Antimony amounts of different *P. somniferum* cultivars' seeds.

Sb levels of seeds ranges between 0.010 mg/kg (Ofis 96) and0.034 mg/kg (Ofis 8).

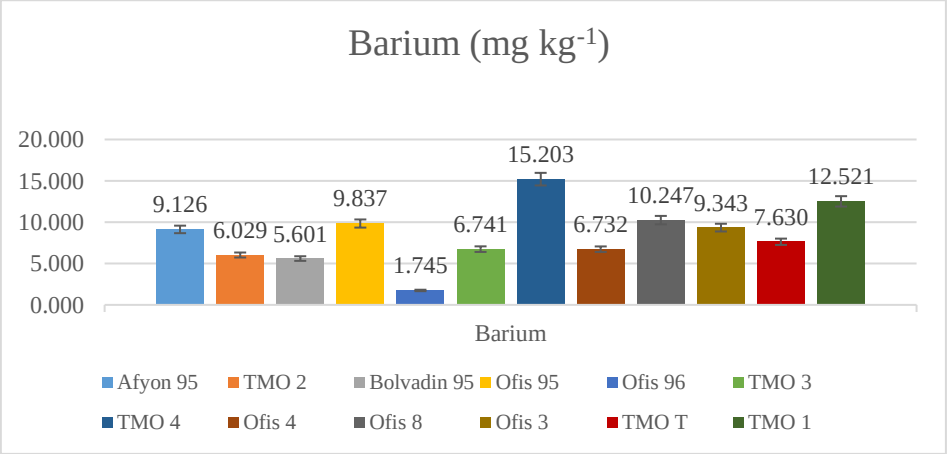

Figure 3.51 Barium amount in measured seeds of different *P. somniferum* cultivars

According to Chaudhry et al. (1977), Ba levels are between 4-50 mg/kg in plants. In this study it is observed that Ba concentrations fluctuates between 1.745 (Ofis 96) and 15.203 mg/kg (TMO 4).

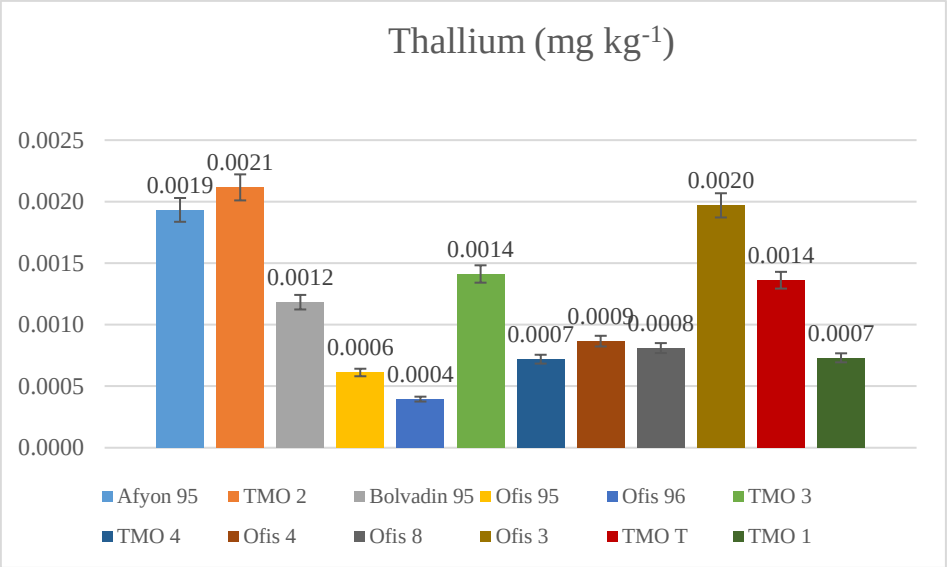

Figure 3.52 Thallium amount in measured seeds of different *P. somniferum* cultivars

As a trace element, Tl concentrations are very low compared to other elements. Highest level of Tl concentration was measured in TMO 2 (0.0021 mg/kg) cultivar.

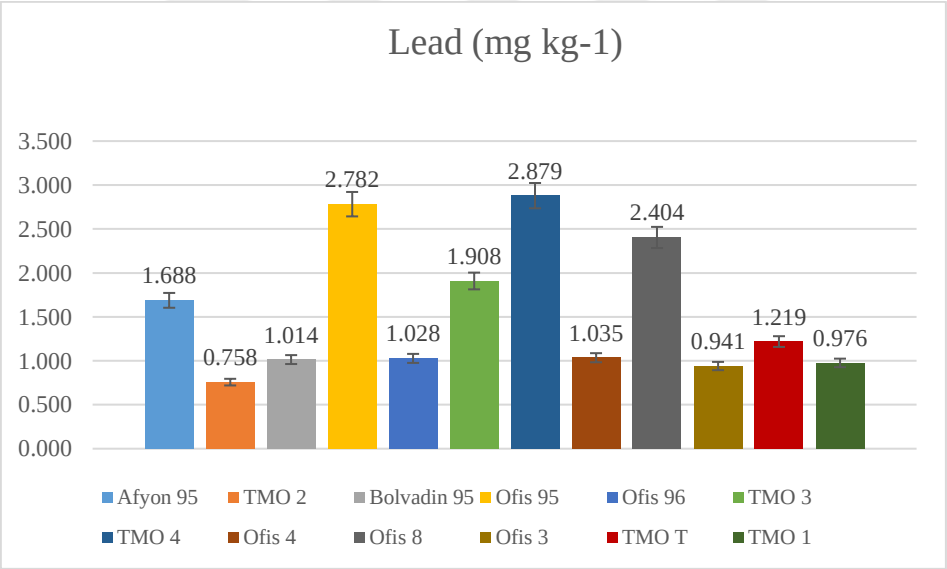

Figure 3.53 Lead amount in measured seeds of different *P. somniferum* cultivars

Pb concentrations are between 0.758-2.879 mg/kg. TMO 4 Pb content determined as the highest while TMO 2 the lowest.

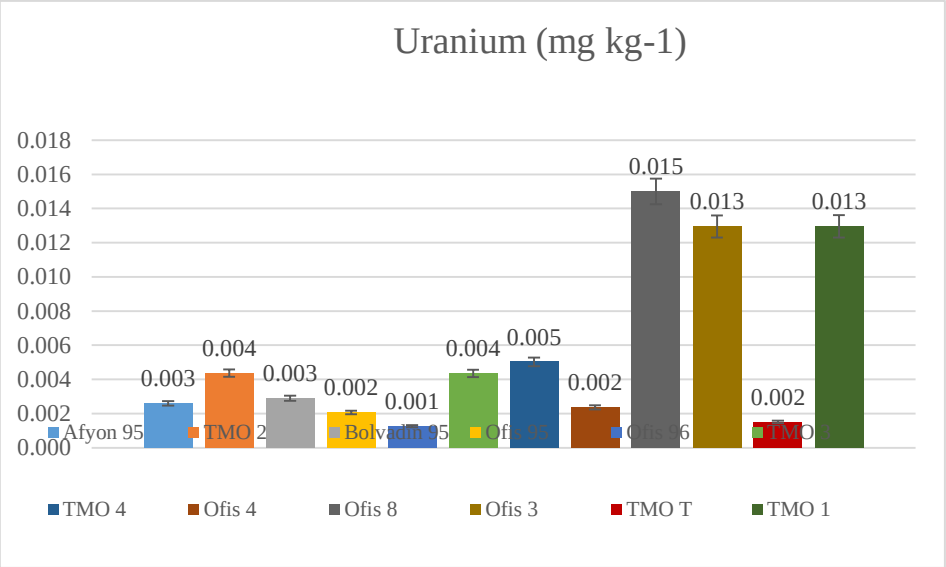

Figure 3.54 Uranium amount in measured seeds of different *P. somniferum* cultivars

All uranium levels are near zero-concentration. Highest U amount found in Ofis 8 (0.015 mg/kg) while lowest in Ofis 96 (0.001 mg/kg). Gramss and Voigt (2015) determined U amount of pea seeds and their results show similarity to this study.

Statistical Analysis

Figure 3.55 When the dendogram formed after hierarchical cluster analysis was examined, clustering analaysis between group average linkage method revealed mainly four clusters. Group A consists of; TMO 3, TMO 1, Ofis 95, TMO 4 and Ofis 8; group B; Ofis 4 and Ofis 3; group C; TMO 2 and Ofis 96 and finally group D Afyon 95 and TMO T. Bolvadin 95 differentiates from all clusters and has a close relation with only TMO2 and Ofis 96. The affinity between Afyon 95 and TMO T which merges with other clusters later, is stronger that the affinity between other 10 cutivars.

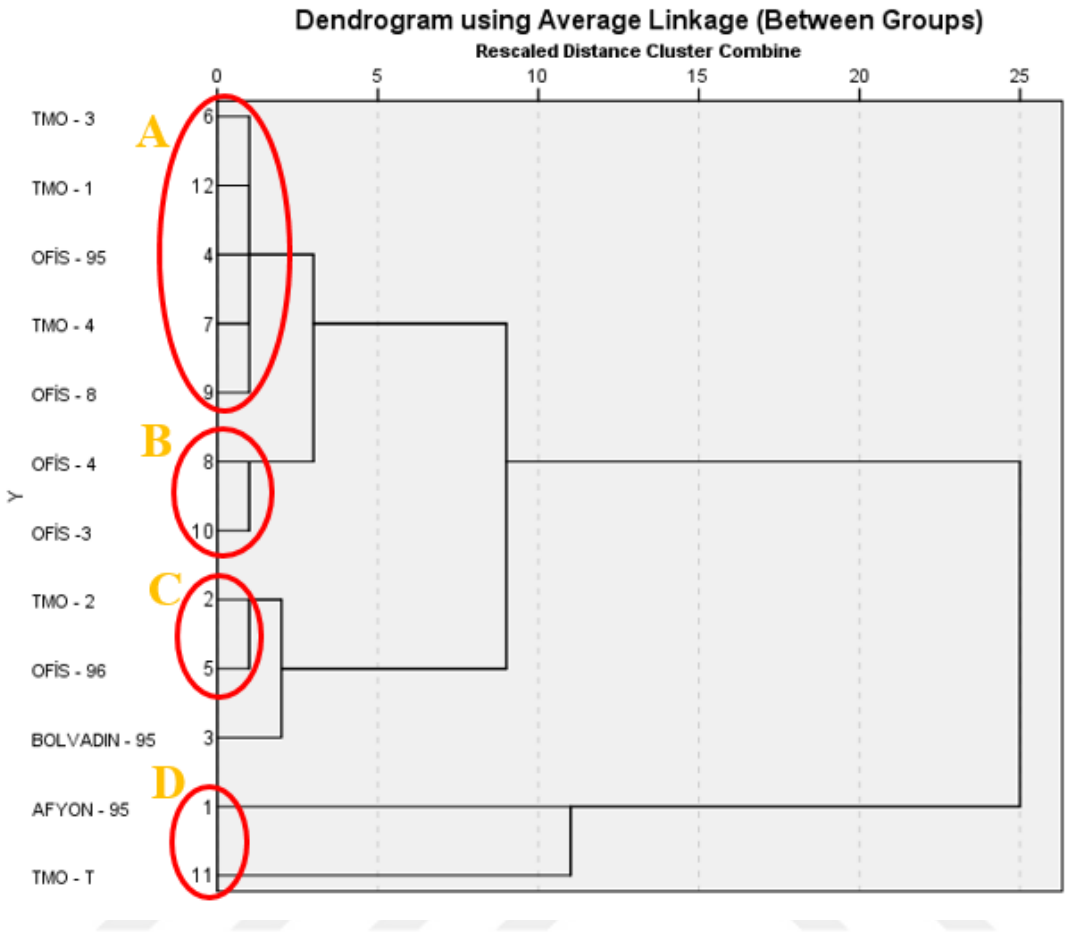

Figure 3.55 Hierarchical cluster dendogram of chemical compounds of opium poppy seeds.

3.2 Phylogenetic Relationships and Genetic Diversity of *P. somniferum*

Phylogenetic relationships and genetic diversity analyses of *P. somniferum* cultivars were conducted by using ITS1 and *trnL- trnF* intergenic spacer sequences and ISSR data, respectively. The data obtained as a result of these analyzes are examined in detail below.

3.2.1 Genetic diversity analyses

ISSR molecular markers were used for reveal the genetic diversity among *P. somniferum* cultivars. 18 ISSR primers were applied in 12 varieties. Distinguishable and visible bands were obtained from 10 primers. After the gel electrophoresis and visualization amplified

DNA fragments bands were scored as 0 for absence and 1 for presence. Some bands were ignored because they were not seen very well. The results of ISSR markers application were shown in Table 3.1. 93 bands were acquired and 86 of them are polymorphic. The average number of bands per primer is 9.3. The band size range was observed as 100-1600 bp long.

Table 3.1 The details of ISSR primers used for analysis of all genotypes of *P. somniferum* cultivars, including primer names, band data, and percentages of polymorphic bands

No	Name	Amplicon Size (bp)	Total number of			(n=12) Polymorphism (%)
			Amplified bands	Monomorphic Bands	Polymorphic Bands	
1	UBC811	350-1300	8	2	6	75
2	UBC817	400-1600	12	0	12	100
3	UBC818	250-1200	8	2	6	75
4	UBC820	250-1300	7	2	5	71.4
5	UBC823	300-1300	10	0	10	100
6	UBC827	200-1300	8	0	8	100
7	UBC857	100-700	8	2	6	75
8	UBC841	150-1200	11	0	11	100
9	UBC847	300-1300	11	0	11	100
10	UBC 855	250-1500	10	0	10	100
Total :			**93**	**7**	**86**	**92.47**

The percentage of polymorphic loci was found overall as 92.47%. The mean of effective number of alleles (ne), observed number of alleles (na), Nei's (1973) gene diversity (h), and Shannon's information index (I) were observed as 1.648, 1, 925, 0.528 and 0.362, respectively showed in Table 3.2.

Table 3.2 The summary of genetic variation statistics for all loci by using diploid ISSR data set with Popgene 32 software

Locus	na*	ne*	h*	I*
Mean	**1.925**	**1.648**	**0.362**	**0.528**
Standart Deviation	**0.265**	**0.334**	**0.159**	**0.209**

na: Observed number of alleles, ne: Effective number of alleles [Kimura and Crow (1964)], h: Nei's (1973) gene diversity, I: Shannon's information index [Lewontin (1972)]

According to PCA, it was observed that analyzed cultivars were clustered two main groups namely as A and B. Group A consist of TMO 1, TMO T and Ofis 4 cultivars, the

other cultivars clustered as group B. Group B can be separated two clusters. This separation between major groups A and B can be explain that plant breeding activities may effect the genomic variation of these varieties resulted in this phylogenetic distributions in present study. Paticularly some gentoypes such as TMO 1 and Ofis 95 were isolated than other genotypes, suggesting that their genomic rearregements due to mutations, genomic recombinations etc.

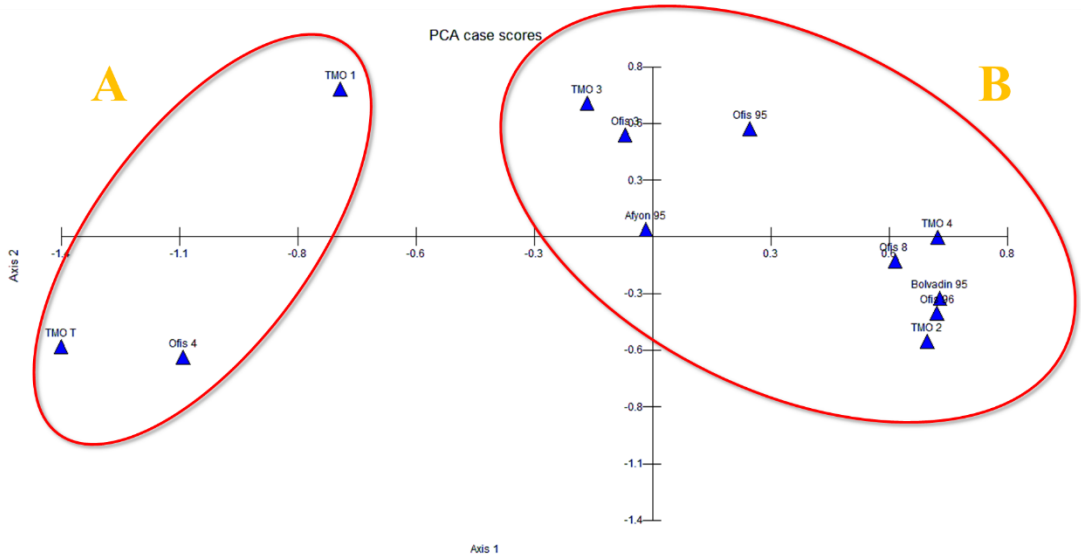

Figure 3.56 Principal Component Analysis (PCA) of *P. somniferum* genotypes generated by using MVSP 3.22. The blue triangles show the plant genotypes and major groups, respectively. The numbers on the axis1 and 2 indicate PCA scores.

3.2.2 Phylogenetic analyses

3.2.2.1 Internal Transcribed Spacer 1 (ITS1)

After PCR reactions, amplicons were migrated in 1.2% agarose gel and were generated single bands in size of 250-350 bp long. After the sequencing process, raw sequences were aligned and edited. Length of edited sequenced are 239 bp and only include ITS1 sequence of ITS region. Images of ITS 1 bands in agarose gel electrophoresis are shown in Figure 3.56.

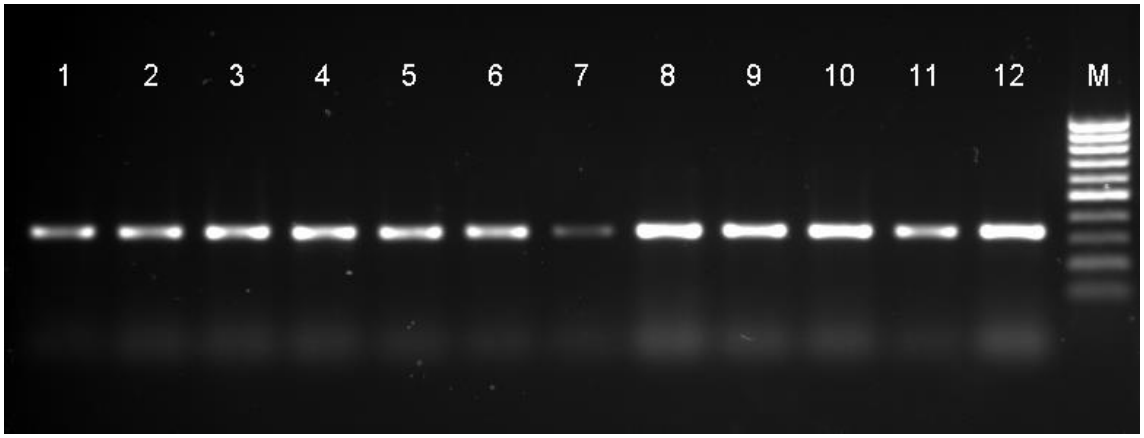

Figure 3.57 The picture of agarose jel of ITS 1 amplicons (M: 100-1000bp DNA ladder, 1: Afyon 95, 2: TMO 2, 3: Bolvadin 95, 4:Ofis 95, 5:Ofis 96, 6:TMO 3, 7:TMO 4, 8: Ofis 4, 9: Ofis 8, 10: Ofis 3, 11: TMO T, 12:TMO 1 genotype's names)

Phylogenetic analysis of opium poppy genotypes was performed and shown in Figure 3.57. The two major groups were identified named as group A and group B. While group A contained 10 genotypes (Afyon 95, Bolvadin 95, TMO 2, Ofis 8, Ofis 96, TMO T, Ofis 3, Ofis 4, Ofis 95 and TMO 1), group B contained two genotypes (TMO 3 and TMO 4). Base on bootstrap values, weak realitonship among genotypes were found ranged from 17% to 77%. The highiest bootstrap value was found as 77% between Afyon 95 and Bolvadin 95, in contrast the lowest bootstrap value was found as 17% between Ofis 95 and Ofis 1. The weak phylogenetic relationships among genotypes may be related with the plant breeding history. Also, the level of genetic diversity (0.528 and 0.362 for Nei's and Shannon's information index, respectively) proved the these data.

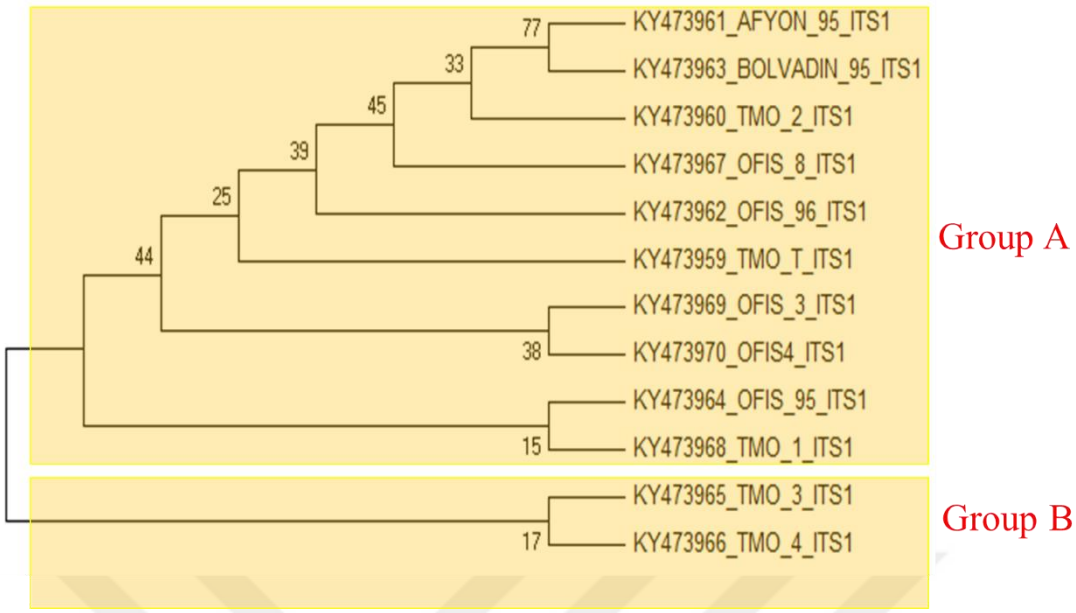

Figure 3.58 Phylogenetic distribution of opium poppy cultivars using ITS1 sequences. Phylogenetic tree was constructed by MEGA7 software using Maximum likelihood (ML) method for 1000 bootstraps. Number on the branches show the bootstrap values.

Sequences were submitted to NCBI GenBank database and acquired the accession numbers, and ITS1 sequences were searched in nucleotide collection of NCBI to find and compare similar sequences by using NCBI MegaBlastn Suite. Top three similar sequences in results of MegaBlastn are which are shown in Table 3.3.

Table 3.3 The details of top three similar ITS1 sequences to *P. somniferum* cultivars obtained from NCBI GenBank

Sequence name	Accession numbers	Organism	Accession numbers	Query Cover (%)	Identity (%)
TMO T	KY473959	*P.somniferum* subsp. *setigerum*	JN584660	99	92
		P.somniferum subsp. *setigerum*	JN584656	99	92
		P.somniferum	DQ250306	99	92
TMO 2	KY473960	*P.somniferum* subsp. *setigerum*	KJ468102	99	94
		P.somniferum subsp. *setigerum*	AY232778	99	94
		P.somniferum	AY232779	99	94
Afyon 95	KY473961	*P.somniferum*	DQ250306	99	97
		P.somniferum	DQ250279	99	97
		P.somniferum cultivar *Nigrum*	DQ364699	99	97
Ofis 96	KY473962	*P.somniferum* subsp. *setigerum*	JN584660	99	93
		P.somniferum subsp. *setigerum*	JN584656	99	93
		P.somniferum	DQ250306	99	93
Bolvadin 95	KY473963	*P.somniferum* subsp. *setigerum*	JN584660	99	96
		P.somniferum subsp. *setigerum*	JN584656	99	96
		P.somniferum cultivar *Nigrum*	DQ364699	99	96
Ofis 95	KY473964	*P.somniferum* subsp. *setigerum*	JN584660	99	92
		P.somniferum subsp. *setigerum*	JN584656	99	92
		P.somniferum	DQ250306	99	92
TMO 3	KY473965	*P.somniferum* subsp. *setigerum*	JN584660	99	92
		P.somniferum subsp. *setigerum*	JN584656	99	92
		P.somniferum	DQ250306	99	92
TMO 4	KY473966	*P.somniferum* subsp. *setigerum*	JN584660	99	92
		P.somniferum subsp. *setigerum*	JN584656	99	92
		P.somniferum	DQ250306	99	92
Ofis 8	KY473967	*P.somniferum* subsp.*setigerum*	JN584660	99	94
		P.somniferum subsp.*setigerum*	JN584656	99	94
		P.somniferum	DQ250306	99	93
TMO 1	KY473968	*P.somniferum* subsp.*setigerum*	JN584660	99	92
		P.somniferum subsp.*setigerum*	JN584656	99	92
		P.somniferum	DQ250306	99	92
Ofis 3	KY473969	*P.somniferum* subsp.*setigerum*	JN584660	99	93
		P.somniferum subsp.*setigerum*	JN584656	99	93
		P.somniferum	DQ250306	99	93
Ofis 4	KY473970	*P.somniferum* subsp.*setigerum*	JN584660	99	92
		P.somniferum subsp.*setigerum*	JN584656	99	92
		P.somniferum	DQ250306	99	92

The joining tree (Figure 3.85) was constructed by using both ITS1 sequences obtained from this study and retrieved from NCBI GenBank database. The tree was constructed for reveal phylogenetic relationships among *P. somniferum* and same/other species at intraspecific level by using ML method. While the eight of 12 genotypes were located with together (bootsrap value as 66%), the other four genotypes (TMO 2, Ofis 8, Afyon 95 and Bolvadin 95) were grouped with other opium poppy genotypes obtained from NCBI with bootsrap value 63%. These four genotypes (TMO 2, Ofis 8, Afyon 95 and Bolvadin 95) seperated from other genotypes, suggesting that these genotypes may originate different ancestor or may expose different plant breeding processes. Also, genomic forces such as mutations, recombinations, reproductions types etc. may induced this seperations.

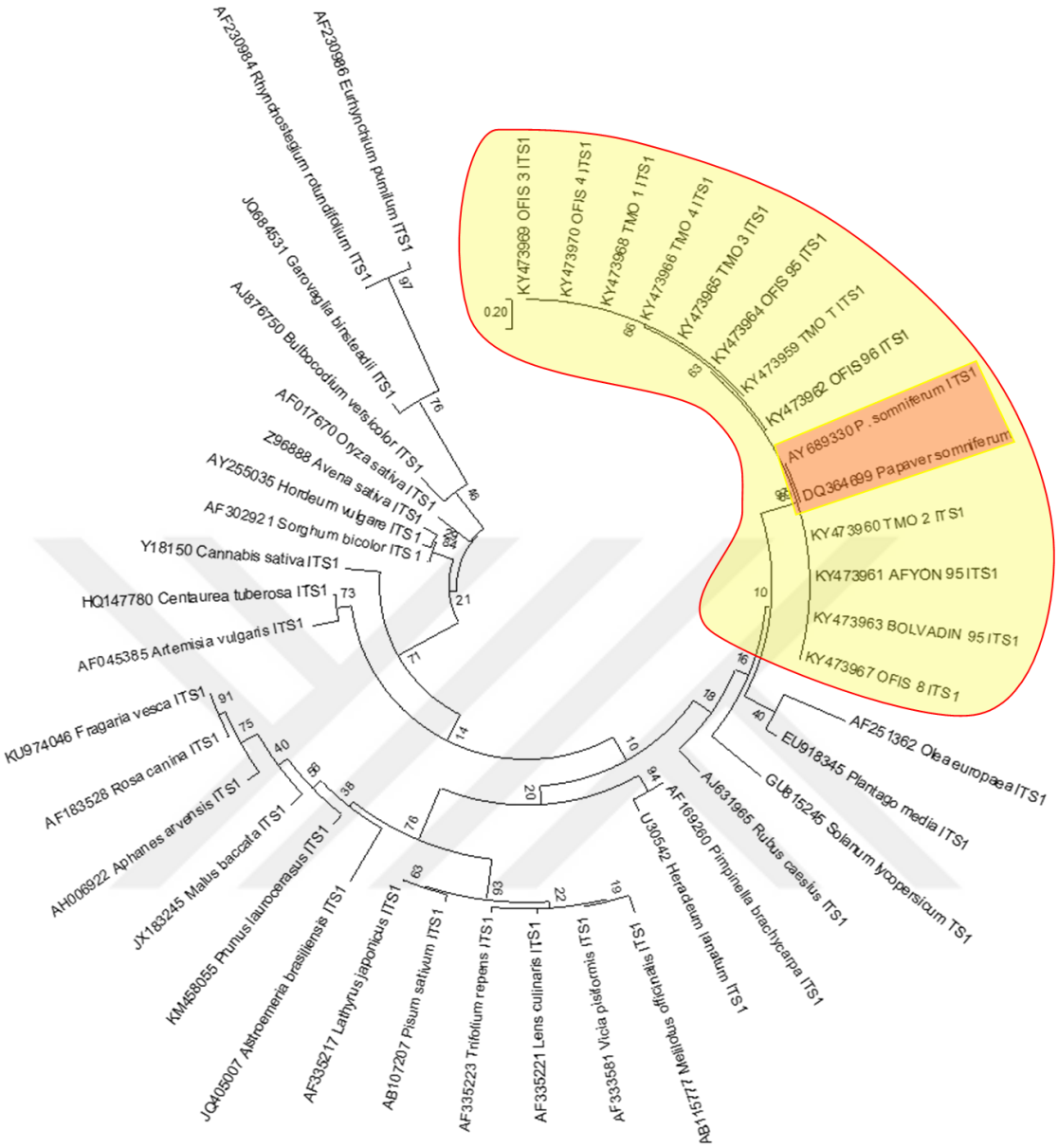

Figure 3.59 The joining phylogenetic tree of ITS1 sequences with *P. somniferum* and other plant species retrieved from NCBI GenBank database. Yellow cluster shows the obtained ITS1 sequences from this study and the red cluster shows other *P. somniferum* ITS1 sequences retrieved from NCBI.

3.2.2.2 *trnL- trnF* Intergenic Spacer

trnL- trnF intergenic spacer region of cpDNA is second phylogenetic marker used in this study for revealing phylogenetic relationships. Previous procedures were applied for *trnL- trnF* intergenic spacer region as applied for ITS1. *trnL- trnF* intergenic spacer DNA regions were amplificated and 450-500 bp long amplicons were obtained. The bands formed by the *trnL- trnF* intergenic spacer amplicons in the agarose gel are shown in the Figure 3.59

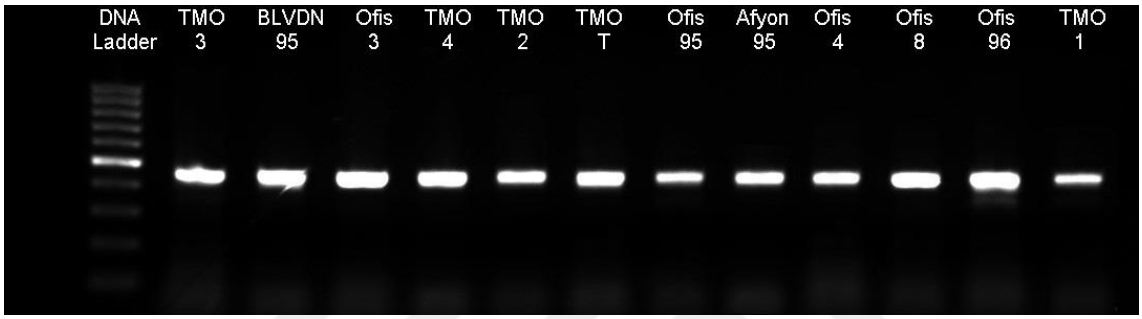

Figure 3.60 *trnL-trnF* intergenic spacer bands in agarose gel (M: 100-1000bp DNA ladder)

After the sequencing process, the raw sequences edited and final sequences submitted to NCBI GenBank database. GenBank accession numbers and some features of *trnL- trnF* intergenic spacer obtained in this study are shown in Table 3.21. Length of *trnL- trnF* intergenic spacer sequences were 453 bp.

The sequences were searched in nucleotide collection of NCBI GenBank database to compare with our sequences. Basic Local Alignment Search Tool (BLASTn) of NCBI was used with "Highly similar sequences (Megablast)" option. Retrieved results of top three sequences per genotype and 12 genotypes sequences numbers which studied in present study, are shown in Table 3.4.

Table 3.4 *trnL-trnF* Intergenic spacer sequences Accession Numbers assigned by GenBank from this study

Sequence name	Accession number	Organism	Accession	Query Cover (%)	Identity (%)
TMO T	KY551571	*P.somniferum*	KU204905	100	99
		P.somniferum	DQ912910	100	99
		P.somniferum subsp.*setigerum*	JN584655	100	99
TMO 2	KY551572	*P.somniferum*	KU204905	100	99
		P.somniferum	DQ912910	100	99
		P.somniferum subsp.*setigerum*	JN584655	100	99
Afyon 95	KY551573	*P.somniferum*	KU204905	100	99
		P.somniferum	DQ912910	100	99
		P.somniferum subsp.*setigerum*	JN584655	100	99
Ofis 96	KY551574	*P.somniferum*	KU204905	100	99
		P.somniferum	DQ912910	100	99
		P.somniferum subsp.*setigerum*	JN584655	100	99
Bolvadin 95	KY551575	*P.somniferum*	KU204905	100	99
		P.somniferum	DQ912910	100	99
		P.somniferum subsp.*setigerum*	JN584655	100	99
Ofis 95	KY551576	*P.somniferum*	KU204905	100	99
		P.somniferum	DQ912910	100	99
		P.somniferum subsp.*setigerum*	JN584655	100	99
TMO 3	KY551577	*P.somniferum*	KU204905	100	99
		P.somniferum	DQ912910	100	99
		P.somniferum subsp.*setigerum*	JN584655	100	99
TMO 4	KY551578	*P.somniferum*	KU204905	100	99
		P.somniferum	DQ912910	100	99
		P.somniferum subsp.*setigerum*	JN584655	100	99
Ofis 8	KY551579	*P.somniferum*	KU204905	100	99
		P.somniferum	DQ912910	100	99
		P.somniferum subsp.*setigerum*	JN584655	100	99
TMO 1	KY551580	*P.somniferum*	KU204905	100	99
		P.somniferum	DQ912910	100	99
		P.somniferum subsp.*setigerum*	JN584655	100	99
Ofis 3	KY551581	*P.somniferum*	KU204905	100	99
		P.somniferum	DQ912910	100	99
		P.somniferum subsp.*setigerum*	JN584655	100	99
Ofis 4	KY551582	*P.somniferum*	KU204905	100	99
		P.somniferum	DQ912910	100	99
		P.somniferum subsp.*setigerum*	JN584655	100	99

Phylogenetic analysis of opium poppy genotypes was performed and shown in Figure 3.61. The two major groups were identified named as group A and group B. While group A contained eight genotypes (Afyon 95, Bolvadin 95, TMO 2, Ofis 96, TMO T, Ofis 3, Ofis 4 and Ofis 95 TMO 1), group B contained two genotypes (TMO 3, Ofis 8, TMO 4 and TMO 1). Base on bootstrap values, weak realitonship among genotypes were found ranged from 5% to 67%. The highiest bootstrap value was found as 67% between Afyon 95 and Ofis 96, in contrast the lowest bootstrap value was found as 5% between TMO 3-Ofis 8 and TMO 4-TMO 1. The weak phylogenetic relationships among genotypes may be related with the plant breeding history. Also, the level of genetic diversity (0.528 and 0.362 for Nei's and Shannon's information index, respectively) proved the these data.

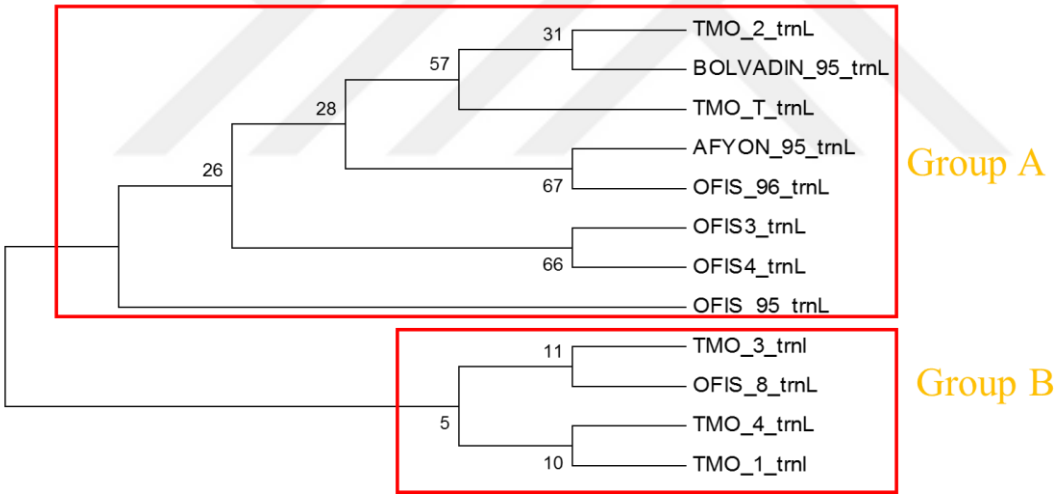

Figure 3.61 Molecular Phylogenetic analysis of present studied *trnL-trnF* intergenic spacer DNA regions by Maximum Likelihood method

The joining tree (Figure 3.85) was constructed by using both *trnL-trnF* sequences obtained from this study and retrieved from NCBI GenBank database. The tree was constructed for reveal phylogenetic relationships among *P. somniferum* and same/other species at intraspecific level by using ML method. While the 11 of 12 genotypes were located with together (bootsrap value as 63%), the other one genotype (TMO 3) was

grouped with other opium poppy genotypes obtained from NCBI. This other one genotype seperated from other genotypes, suggesting that these genotypes may originate different ancestor or may expose different plant breeding processes. Also, genomic forces such as mutations, recombinations, reproductions types etc. may induced this seperations.

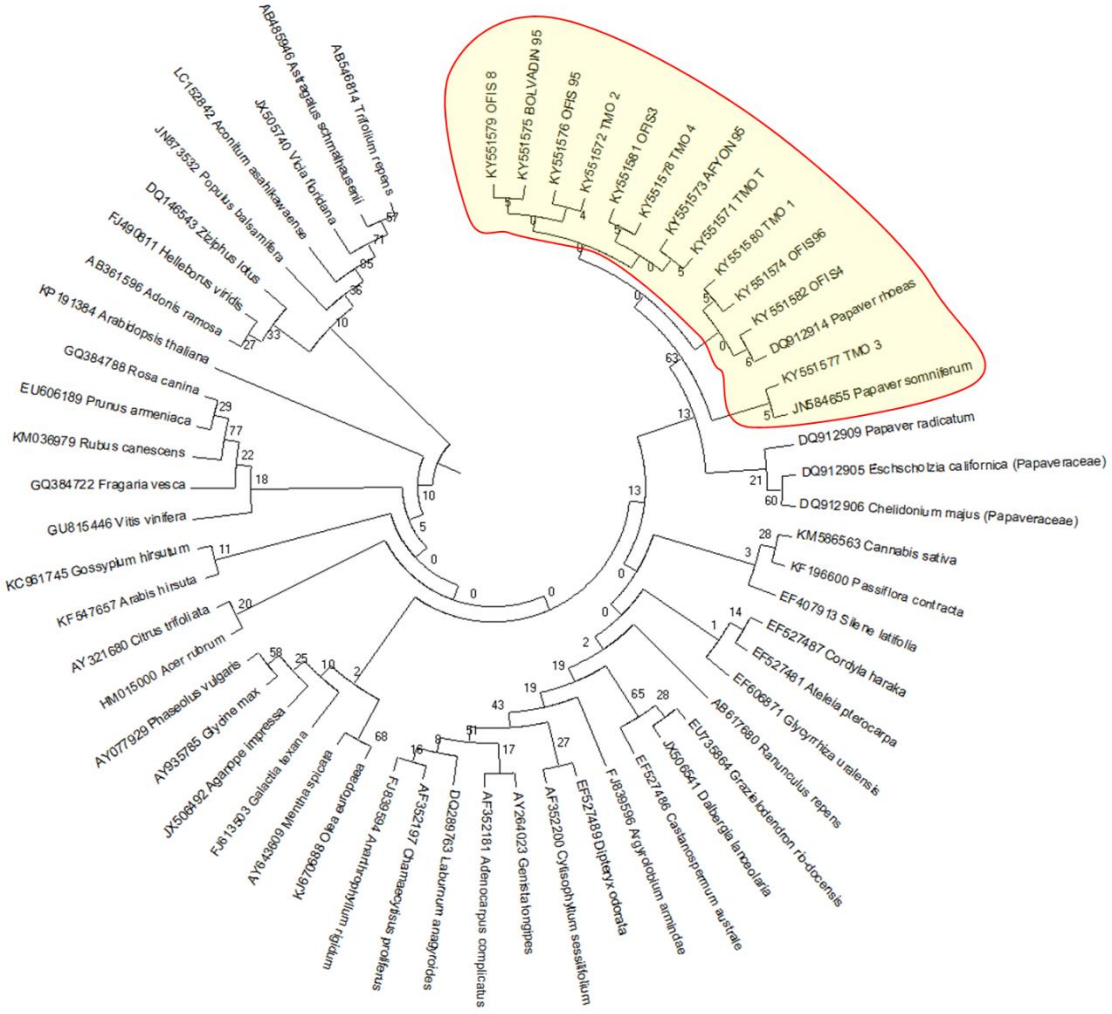

Figure 3.62 The joining phylogenetic tree of *trnL-trnF* intergenic spacer sequences with *P. somniferum* and other plant species retrieved from NCBI GenBank database. Yellow cluster shows the obtained *trnL-trnF* sequences from this study.

CONCLUSION

- In the present study, fatty acids, mineral elements and protein contents, which represent nutritional specifications, were determined in seeds of pharmaceutically and economically important Turkish opium poppy cultivars. Due to its high levels of Linoleic acid, Ca, Mg, Cr, Cu, Fe, Mn, P, K, Se, Zn and Na, it can be said that opium poppy seeds could be used as important food addictives, when compared to recommended daily diatery allowances.
- An incompatibility between hierarchical cluster dendogram and molecular dendogram was observed. Further investigations are required on genes responsible for synthesis pathways of these chemical compounds to find a compatibility between these dendograms.
- Genetic diversity levels of 12 opium poppy cultivars were analyzed by using 10 ISSR markers, and results indicated that this marker system can be efficiently used for opium poppy cultivars.
- The mean genetic diversity levels were found as 0.528 and 0.362 for Nei's and Shannon's methods respectively. It can be proposed that Turkish opium poppy gene pool shows high level of genetic diversity.
- According to the results of this study, it can be concluded that ITS 1 region is more reliable then trnL-F region for phylogenetic analysis, especially for opium poppy cultivars.
- Based on joining tree data of 12 Turkish opium poppy cultivars, ITS 1 region indicated a higher bootstrap value (66%), than *trnL- trnF* (63%) to define phlogenomic positions of 12 Turkish opium poppy cultivars.

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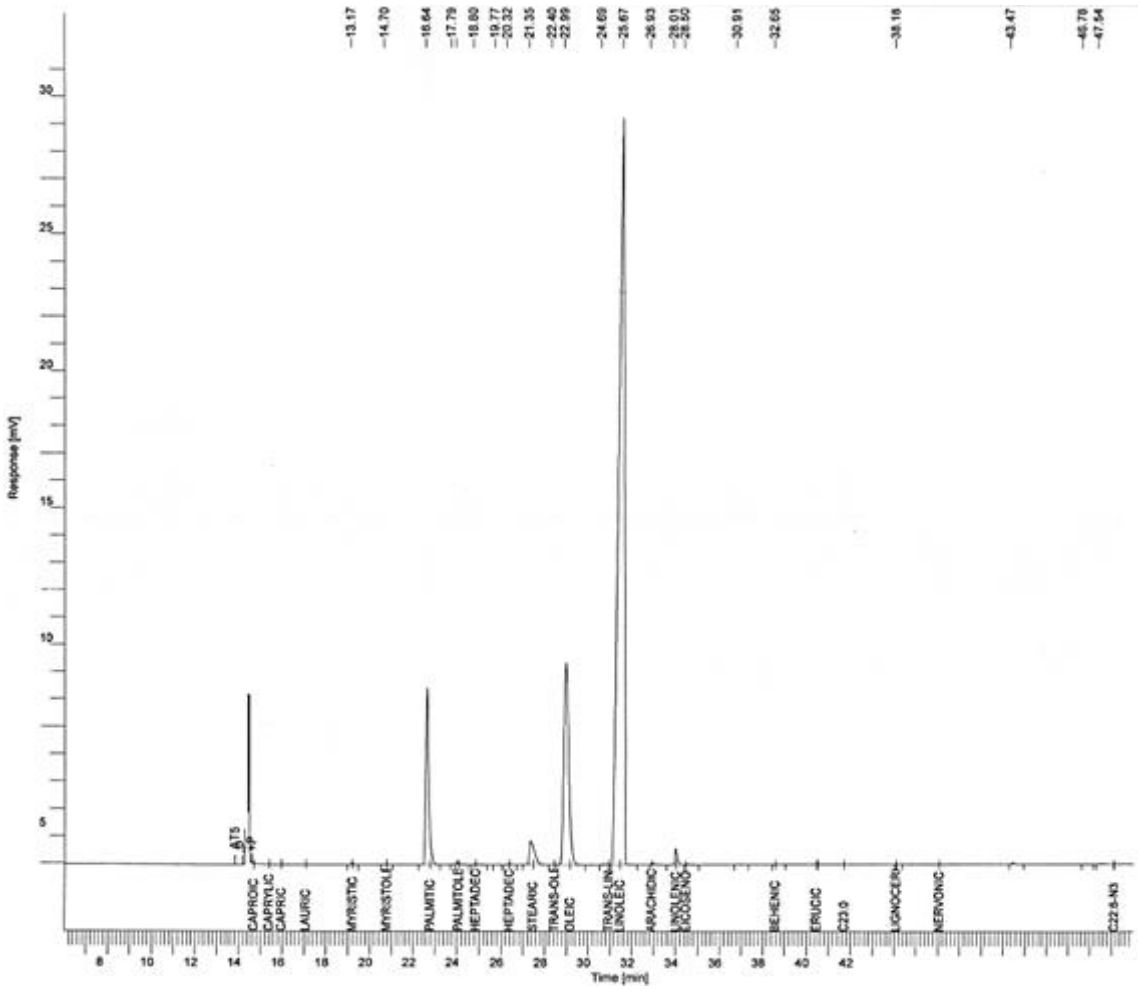
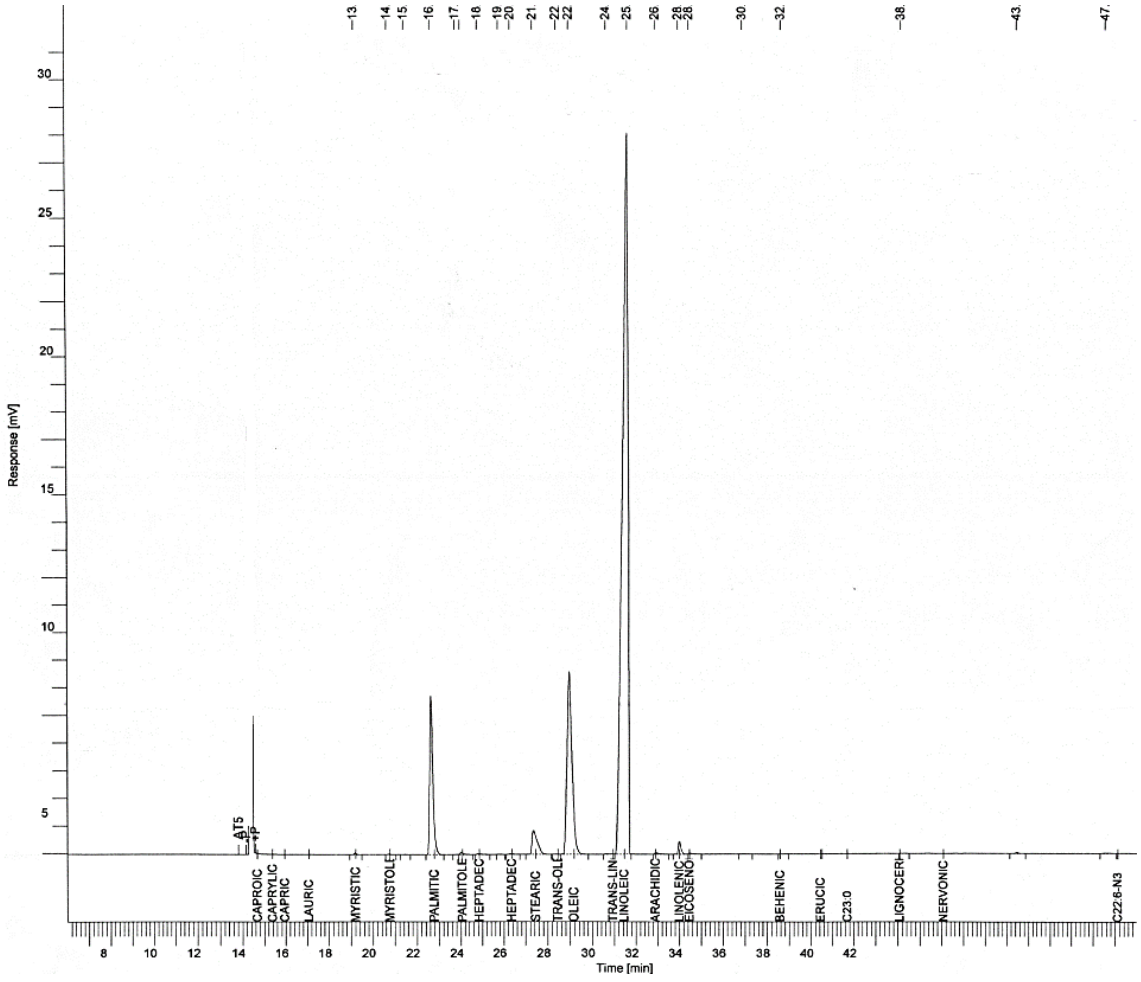
SUPPLEMENTARY

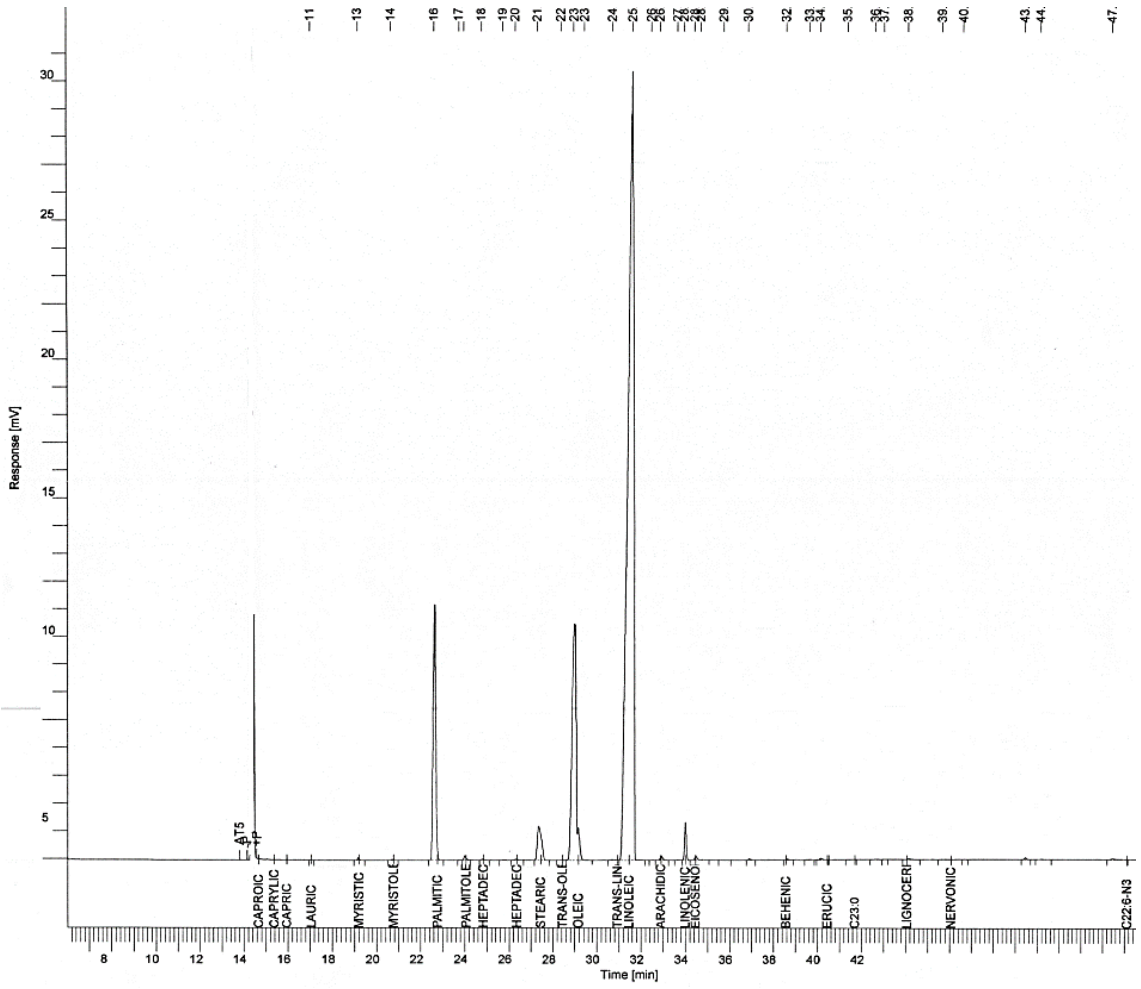
Suplementary Figure 1 GC-FID chromatogram of seeds of *Papaver somniferum* L. (Ofis 8 cultivar), showing various fatty acids.



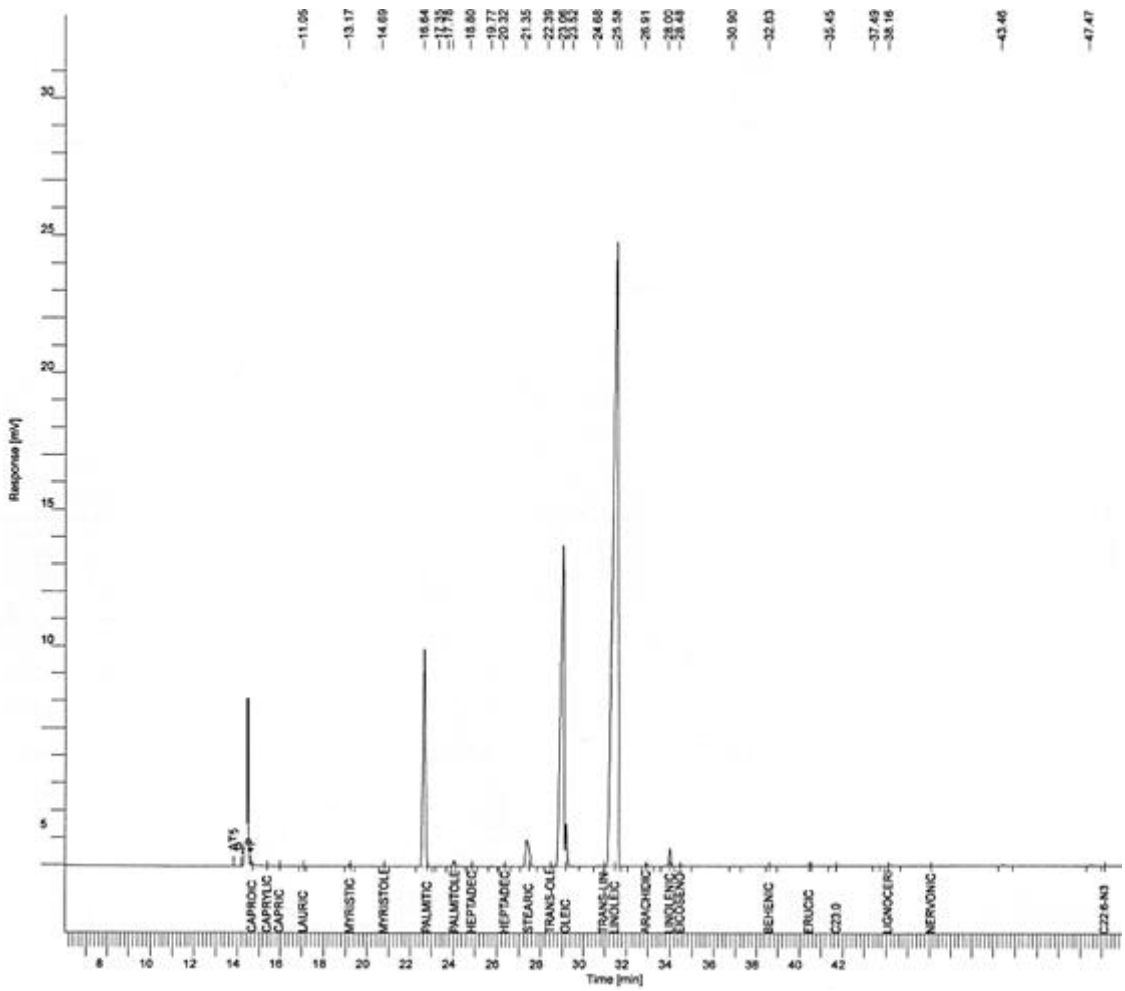
Suplementary Figure 2 GC-FID chromatogram of seeds of *Papaver somniferum* L. (TMO 1 cultivar), showing various fatty acids.



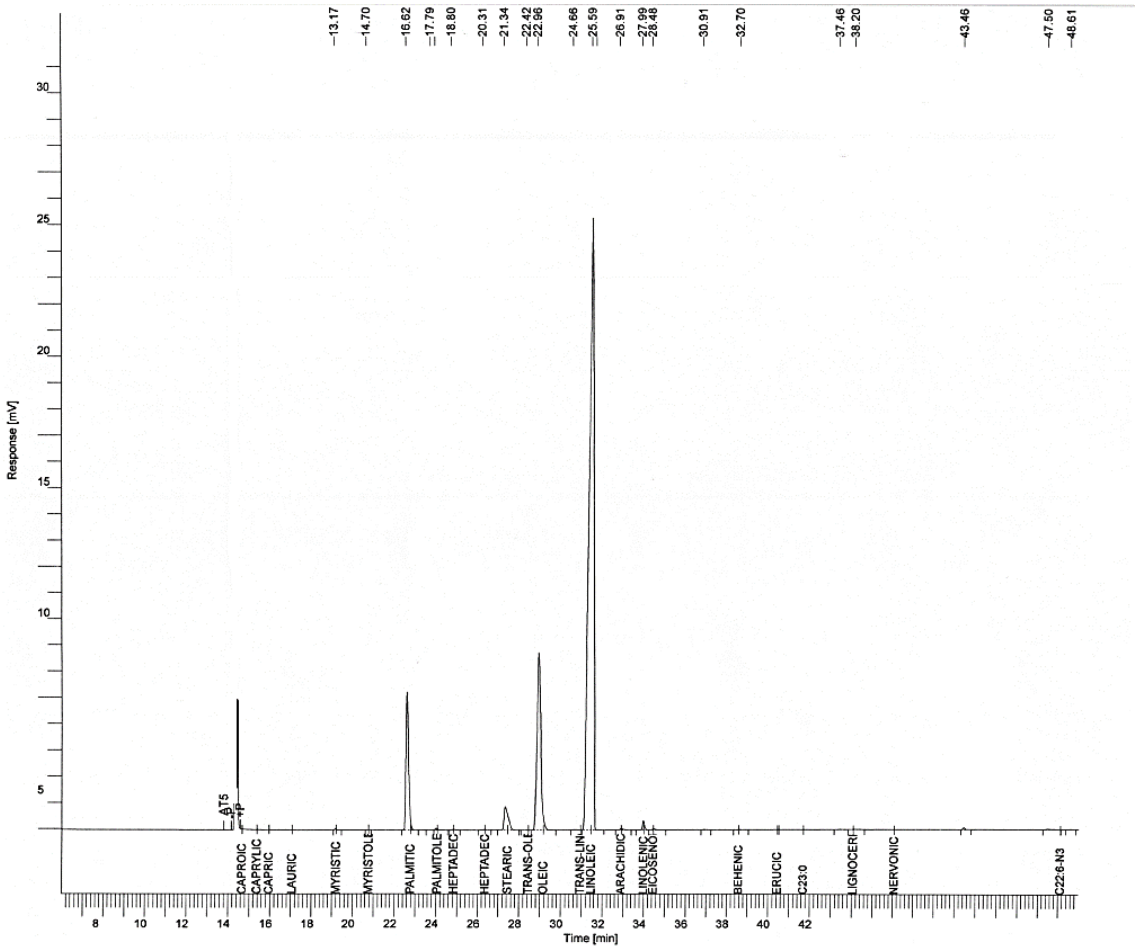
Suplementary Figure 3 GC-FID chromatogram of seeds of *Papaver somniferum* L. (Ofis 3 cultivar), showing various fatty acids.



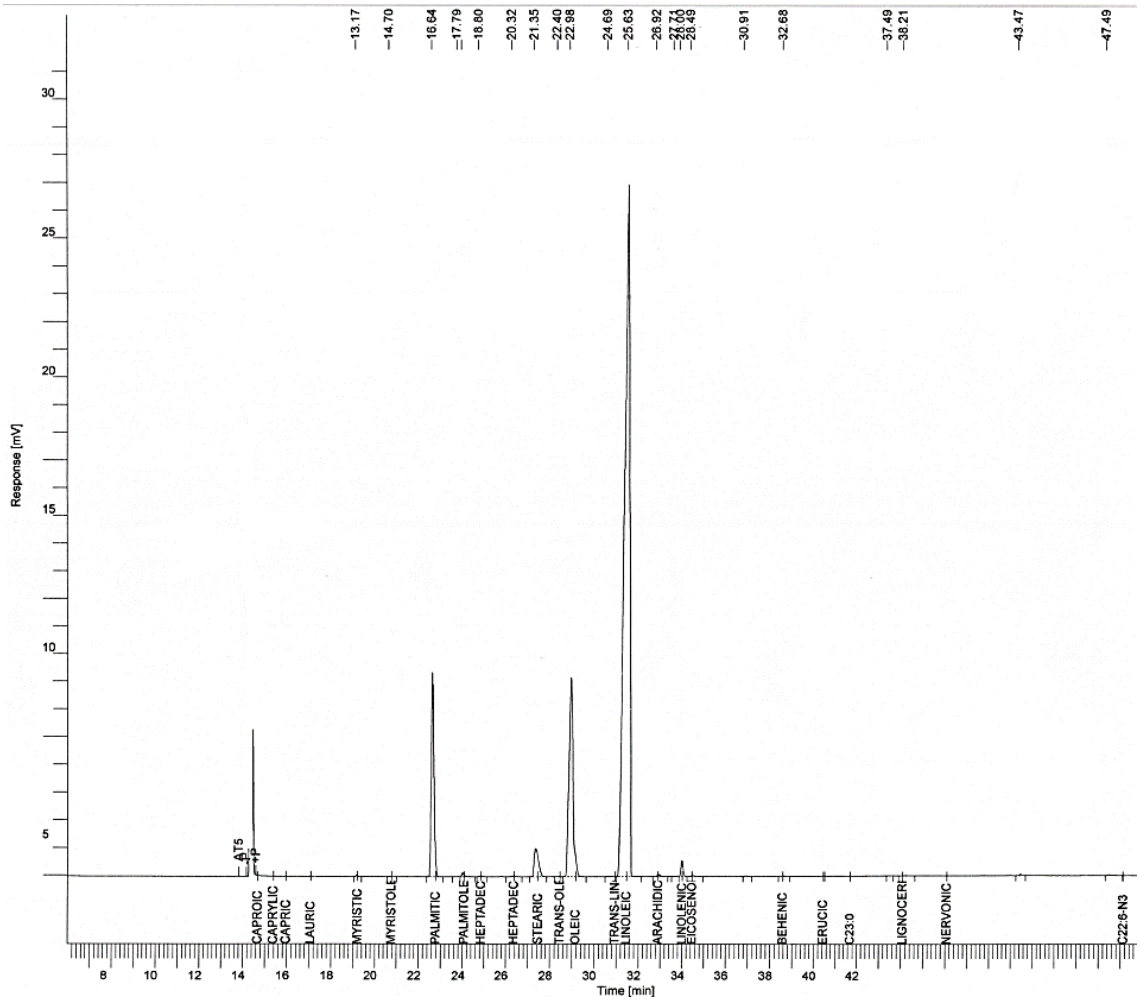
Suplementary Figure 4 GC-FID chromatogram of seeds of *Papaver somniferum* L. (TMO T cultivar), showing various fatty acids.



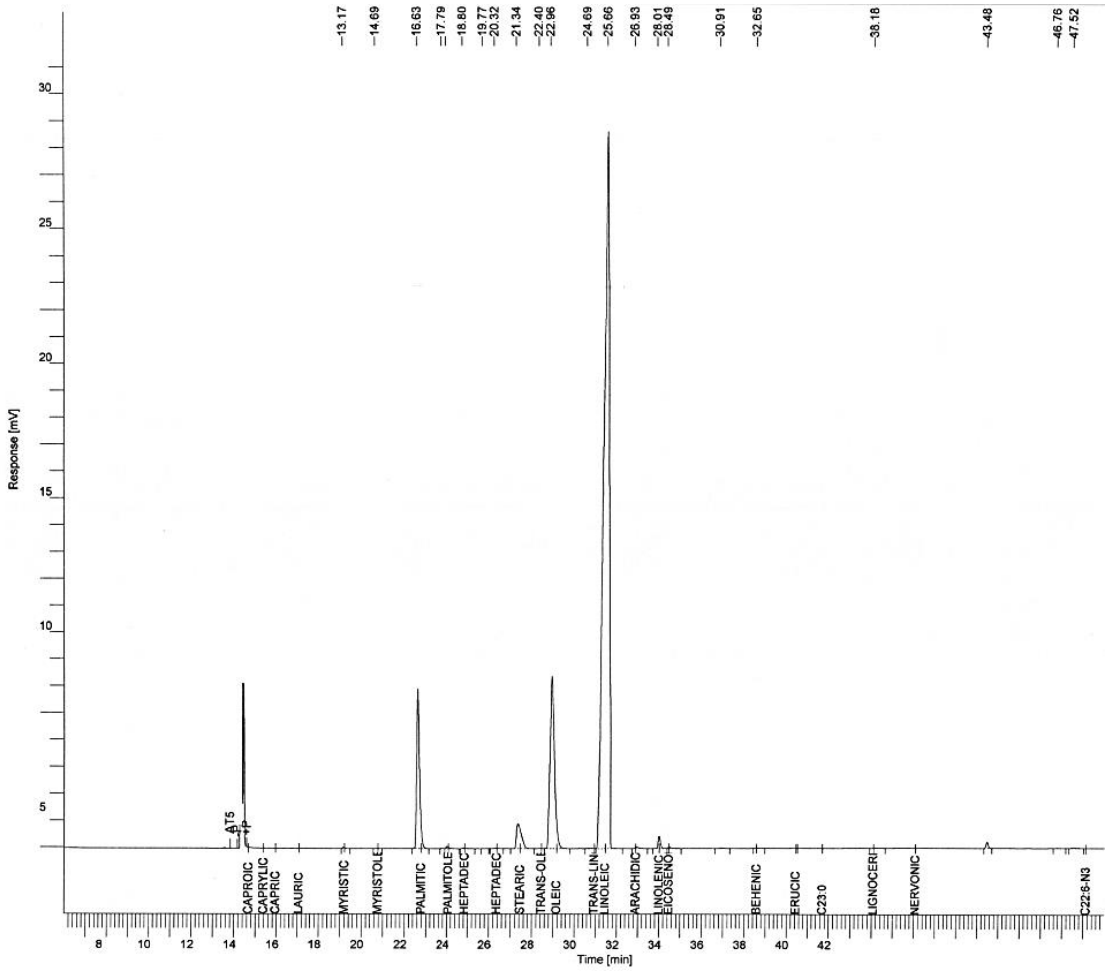
Suplementary Figure 5 GC-FID chromatogram of seeds of *Papaver somniferum* L. (Afyon 95 cultivar), showing various fatty acids.



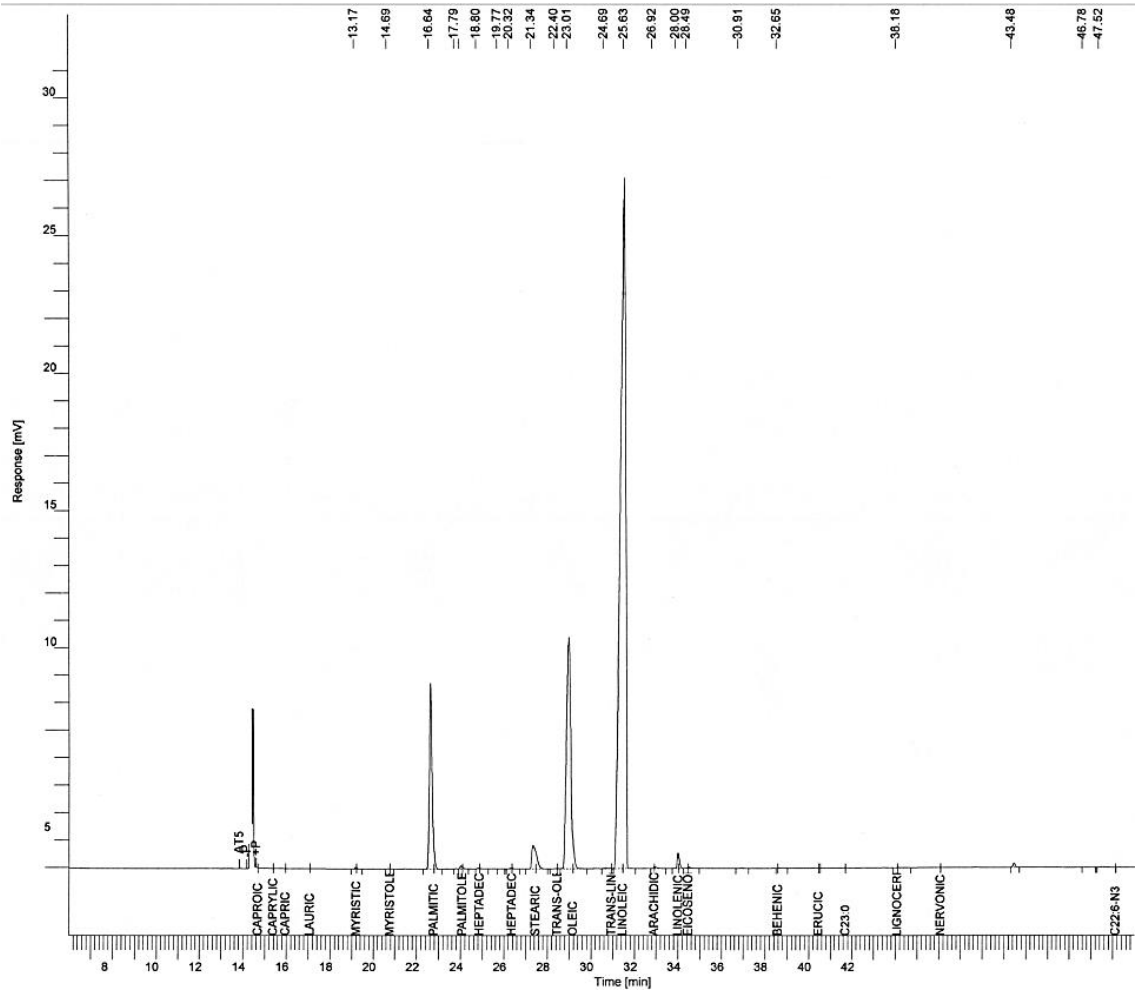
Suplementary Figure 6 GC-FID chromatogram of seeds of *Papaver somniferum* L. (TMO 2 cultivar), showing various fatty acids.



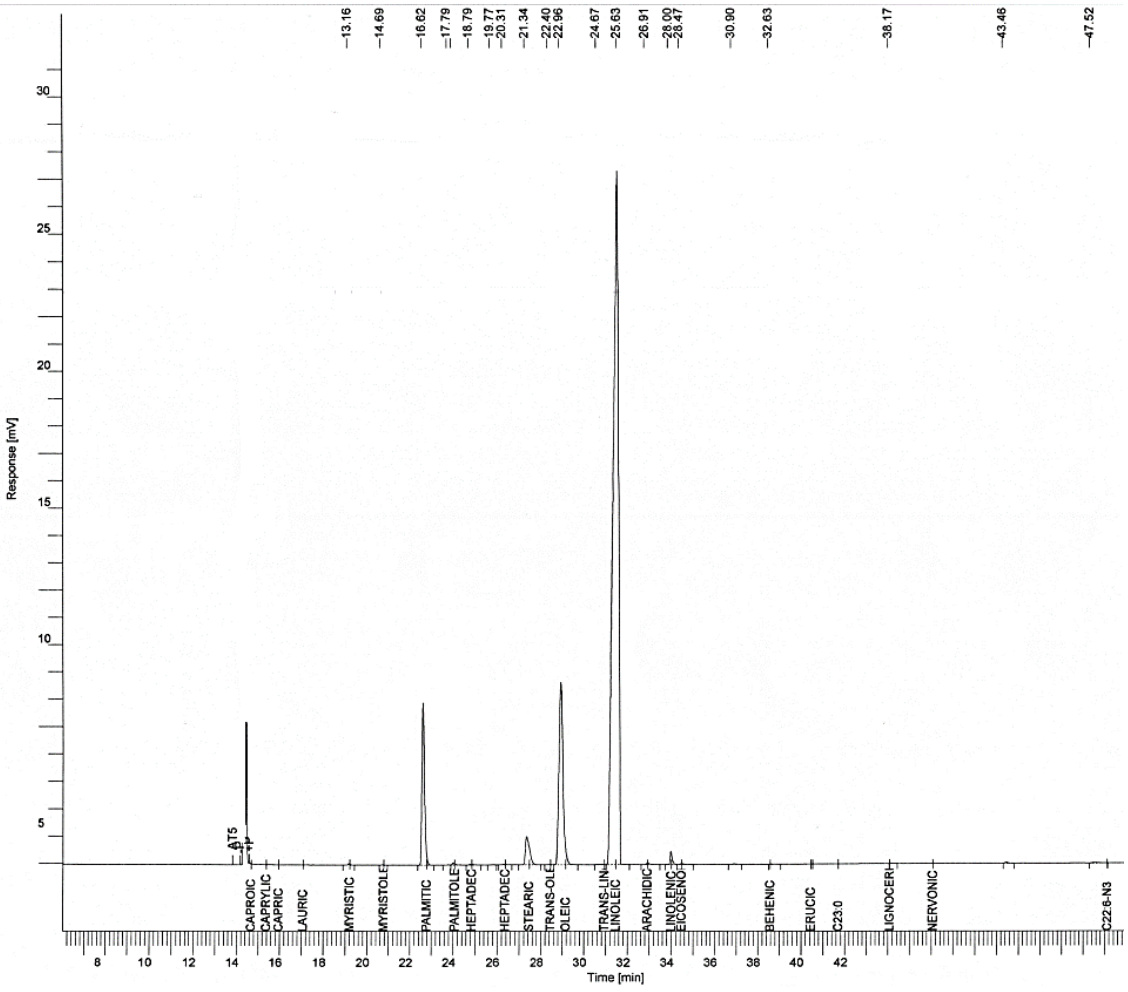
Suplementary Figure 7 GC-FID chromatogram of seeds of *Papaver somniferum* L. (Ofis 95 cultivar), showing various fatty acids.



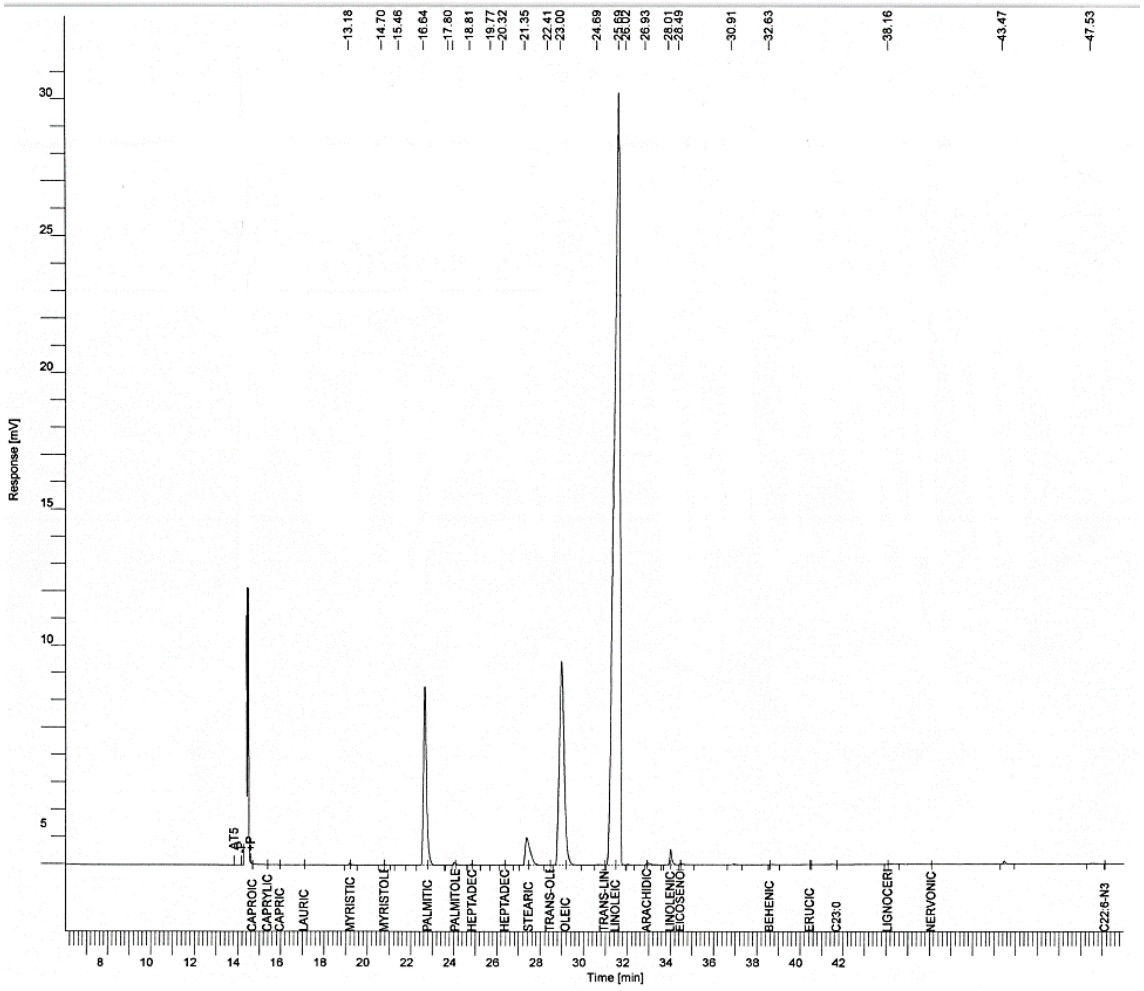
Suplementary Figure 8 GC-FID chromatogram of seeds of *Papaver somniferum* L. (Bolvadin 95 cultivar), showing various fatty acids.



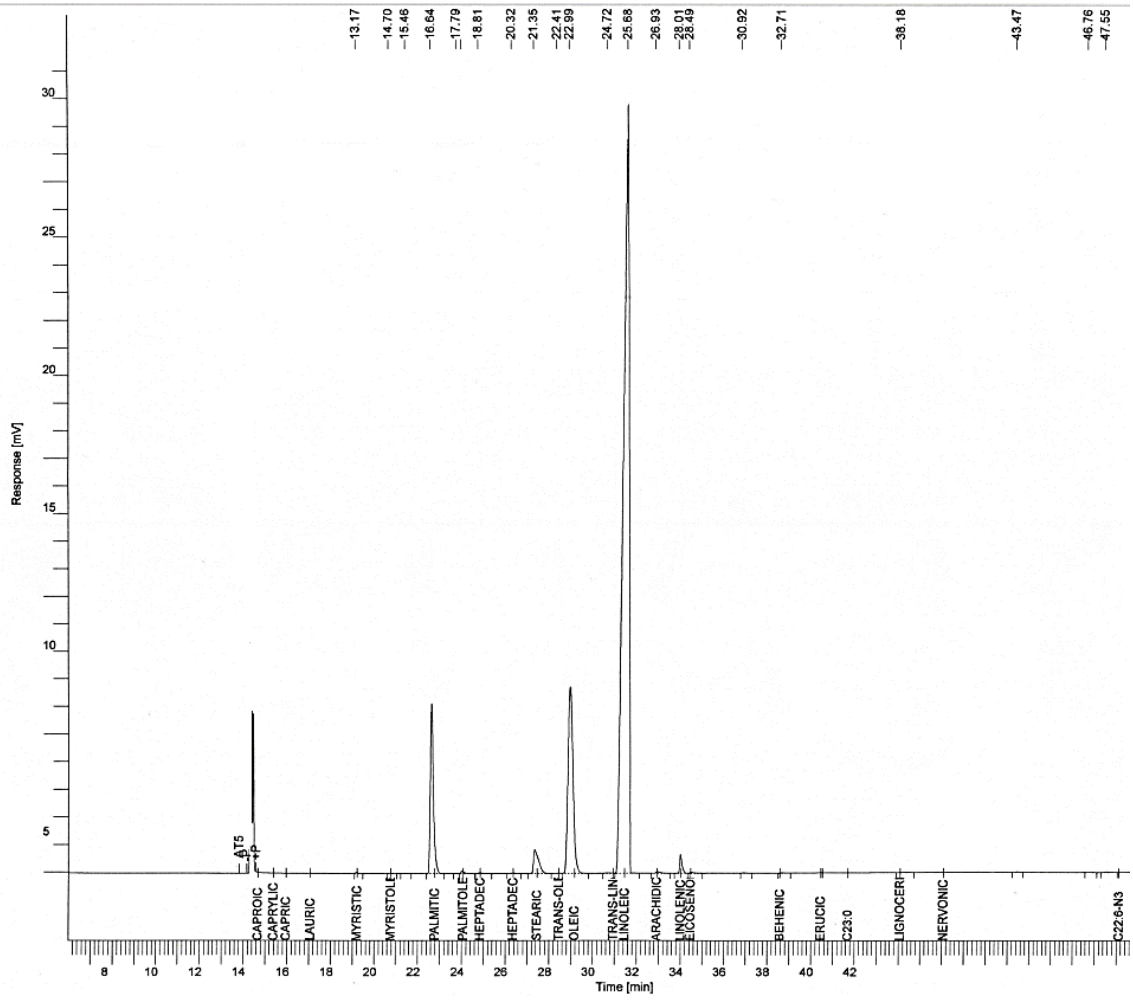
Suplementary Figure 9 GC-FID chromatogram of seeds of *Papaver somniferum* L. (Ofis 96 cultivar), showing various fatty acids.



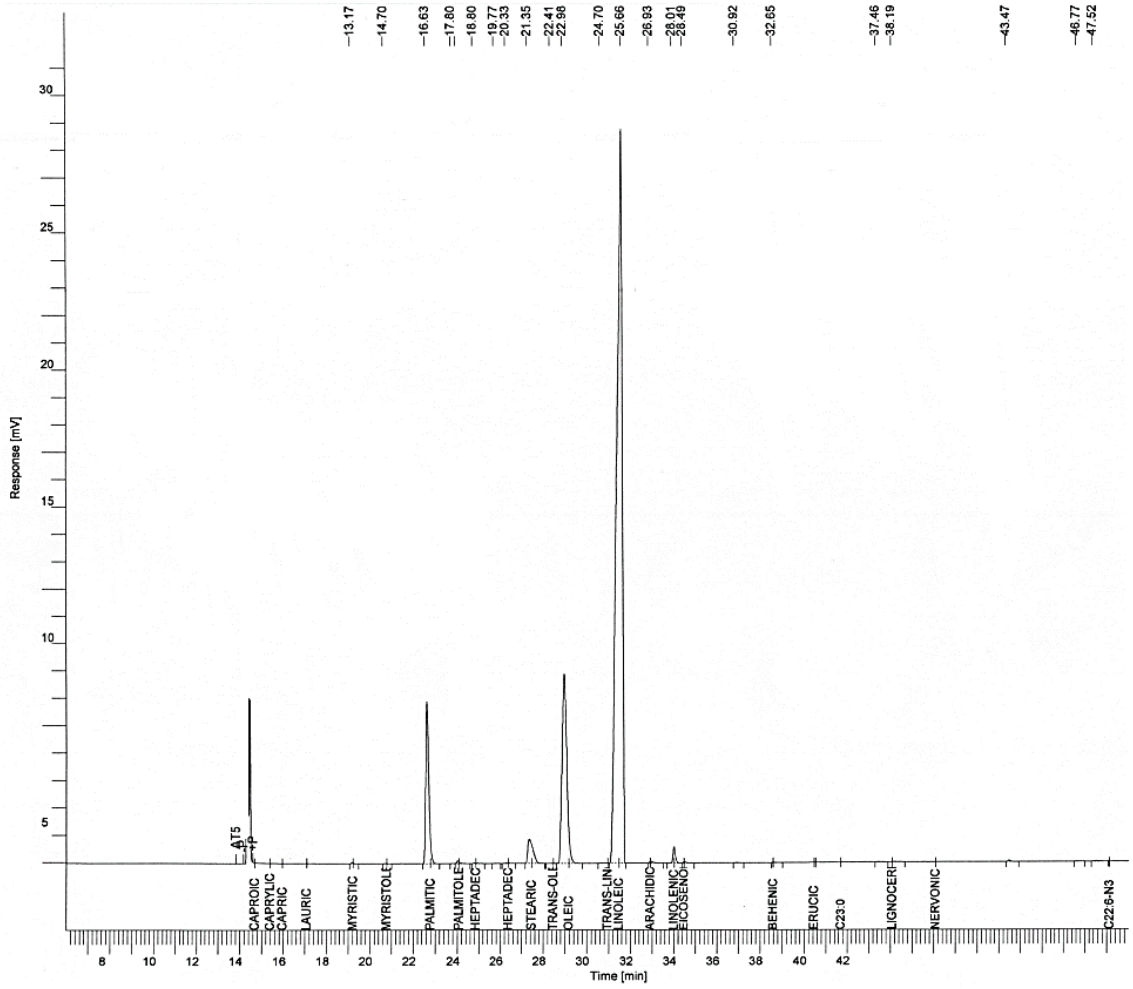
Suplementary Figure 10 GC-FID chromatogram of seeds of *Papaver somniferum* L. (Ofis 4 cultivar), showing various fatty acids.



Suplementary Figure 11 GC-FID chromatogram of seeds of *Papaver somniferum* L. (TMO 4 cultivar), showing various fatty acids.



Suplementary Figure 11 GC-FID chromatogram of seeds of *Papaver somniferum* L. (TMO 3 cultivar), showing various fatty acids.



RESUME

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Papers:

1. Vatansever, R., Koc, I., Ozyigit, I. I., Sen, U., Uras, M. E., Anjum, N. A., and Filiz, E. (2016). Genome-wide identification and expression analysis of sulfate transporter (SULTR) genes in potato (*Solanum tuberosum* L.). Planta, 1-17. DOI 10.1007/s00425-016-2575-6

2. R.Vatansever, M. E. Uras, U. Sen, I. I. Ozyigit, E. Filiz, (2016), Isolation of a transcription factor DREB1A gene from *Phaseolus vulgaris* and computational insights into its characterization; protein modelling, docking and mutagenesis, J Biomol Struct Dyn, 1-12.

3. Filiz, E., Vatansever, R., Ozyigit, I. I., Uras, M. E., Sen, U., Anjum, N. A., and Pereira, E. (2017). Genome-wide identification and expression profiling of EIL gene family in woody plant representative poplar (*Populus trichocarpa*). *Archives of Biochemistry and Biophysics*.