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The Millenium Olives (*Olea europea* L.) in Lebanon: Rejuvenation, Micropropagation and Olive Oil Variation during Maturation and Conservation.

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Dedication

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Résumé

Dans l'objectif à plus long terme de préserver les ressources génétiques locales d'olivier (*Olea europea* L.), ce travail a consisté en une mise au point de la technique d'initiation des cultures à partir des bourgeons axillaires des arbres millénaires de Bchealeh *in vivo* et *in vitro*. Les paramètres de l'huile ont été également étudiés et ont permis la classification de l'huile comme «extra vierge » selon les normes internationales du Conseil Oléicole Internationale.

Les techniques de multiplication ont été précédées par une série de traitements pour la désinfection du matériel introduit. Le traitement le plus propice a été celui composé d'un mélange de fongicides Carbendazim (2g/l) et fosethyl-aluminium (6g/l), de détergents NaOCl (10%) et Chloroxylenol (1.5%), et de 1mg/l de l'Oxytetracycline, avec 56.7% de survivants.

Les explants ont été initiés *in vitro* à partir des pousses de l'année sur les milieux MS, OM et WPM additionnés de BAP (2, 3, 4mg/l). Les taux les plus élevé de débourrement (47%), du nombre de nouvelles tiges/explant (1.35) ainsi que celui du coefficient de multiplication (1.08) ont été obtenus durant la saison d'automne sur les milieux MS suivi respectivement par les milieux OM et WPM. L'explant le plus long (1.78cm) et le nombre de nouvelles feuilles/explant (3.58) ont été observés durant la première subculture sur le milieu MS additionné de 3mg/l de BAP. Tous les paramètres étudiés ont diminué en passant d'une subculture à l'autre. Après une phase d'élongation, le meilleur taux d'enracinement (22%) a été observé sur le milieu additionné de 2mg/l ANA. Durant l'automne, les explants qui ont developpé des cals à leur base ont été transférés sur le même milieu, ceci a permi le développement racinaire de 23% du materiel végétal sans passer par le stade de multiplication.

L'enracinement des boutures de Bchealeh *in vivo* a été influencé par la taille de la bouture, la saison de prélèvement et le milieu utilisé. Le meilleur taux de débourrement a été observé durant le printemps de 2010. Le taux moyen des boutures enracinées en 2010 était 10.67% alors que ce taux n'a pas dépassé les 4% en 2009.

La teneur en huile d'huile (28.96%), le rapport chair/graine (6.63:1) ainsi que le poids (4.31g) les plus élevés ont été enregistrés durant la quatrième semaine de Novembre 2010. Au cours de la maturation, tous les paramètres étudiés ont significativement augmenté, alors qu'une diminution a été observée lorsque la couleur des olives a changé en noir. La composition de l'huile en acide gras a été determinée. L'acide oléique a dominé avec 74.8% suivi respectivement par les acides palmitique (9.45%), linoléique (6.26%), stéarique (4.11%), linoléque (0.48%) et palmitoléque (0.39%).
Un an après sa conservation, l'huile de Bchealeh a respecté les normes du COI et de Libnor avec les valeurs maximales enregistrées pour les indices d'acide (0.41), de peroxide (19.1) et d'iode (87.2).

Mots clés: *Olea europea* L., Bchealeh, *in vivo*, *in vitro*, MS, automne, huile extra vierge.

Abstract

In order to preserve the local genetic resources of olive (*Olea europea* L.), this study was aimed to establish two techniques: culture initiation from axillary buds *in vivo* and *in vitro* of Bchealehs millennium trees. Olive oil parameters were also studied; the recorded results, that were within the IOC trade standards, allowed to classify the oil produced by the millennium trees as extra virgin.

The multiplication techniques were preceded by series of treatments in order to minimize the infections of the newly introduced explants. The propitious treatment which gave the best survival rate (56.7%) *in vitro* was the one composed of Carbendazim (2g/l) and fosethyl-aluminium (6g/l) as fungicides, NaOCl (15%) and Chloroxylenol (2.5%) as detergent, and one antibiotic the oxytetracycline (1mg/l).

Explants were induced *in vitro* from young annual shoots on MS, OM and WPM supplemented with BAP (2, 3 and 4mg/l). The best proliferation rate (47%), newly developed explants (1.35) and multiplication rate (1.08) were obtained during autumn on MS media followed by OM and WPM. The longest explants (1.78cm) and the highest number of newly developed leaves (3.58) were observed during the first subculture on the MS media added with 3mg/l BAP. During subcultures, all the studied parameters decreased. After an elongation phase, MS added with 2mg/l of NAA gave the highest rooted explants (22%). During autumn, explants which developed callus on their base were transferred to fresh media that facilitated the rooting of 23% without passing by any subculture.

The *in vivo* rooting ability of Bchealehs cuttings was influenced by cutting size, collection season and medium used. The highest percentage (88%) of emerged buds was recorded during spring 2010. The mean value of rooted Bchealehs cuttings during 2010 was 10.67%, while this amount didn't exceed 4% during 2009.

The highest oil content (28.96%), flesh/pit ratio (6.63:1), and weight (4.31g) were recorded during the last week of November 2010. During maturation all the studied parameters increased significantly and then a slight decrease was observed when olives turned totally into black and overripe. The fatty acid composition was also determined, and the oil contained oleic acid (74.8%), palmitic acid (9.45%), linoleic acid (6.26%), stearic acid (4.11%), linolenic (0.48%) and palmitoleic (0.39%).

One year after its conservation, Bchealehs oil met the IOOC and Libnor standards for exportation of extra virgin olive oil with maximum recorded value of free acid (0.41); peroxide value (19.1) and iodine value 87.2.

Key words: *Olea europea* L., Bchealeh, *in vivo*, *in vitro*, MS, autumn, extra virgin oil.

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ABBREVIATION LIST

AOAC	Association of Official Analytical Chemists
BAP	6-benzylamino purine
BC	Before Christ
C	carbon
Carb	carbendazim
cm	centimeter
cv	cultivar
°	degree
ºC	degree Celsius
et al.	et alii (mot latin)= and others
EEC	European Economic Community
e.g.	exempli gratia/ for example
EU	European Union
FAO	Food and Agriculture Organization
FFA	Free Fatty Acid
g	gram
GC	gaz chromatography
g/l	gram per litre
h	hour
ha	hectare
HCl	hydrogen chloride
HDL	high-density lipoprotein
h/d	hour per day
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ICl	Iodine mono-chloride
IOOC	International Olive Oil Council
2ip	2-isopentenyl adenine
ISO	International Organization for Standardization

IUPAC	International Union of Pure and Applied Chemistry
Kg	kilogram
L	liter
L.	Linne Charles
LDL	low-density lipoprotein
Libnor	Lebanese Standards Institution
m	meter
µl	microliter
µ	micron
mEq	milliequivalent
mg	milligram
mg/l	milligram per liter
min	minute
ml	milliliter
mm	millimeter
MS	Murashige and Skoog
MS1	MS added with 2mg/l of BAP
MS2	MS added with 3mg/l of BAP
MS2	MS added with 4mg/l of BAP
MUFA	monounsaturated fatty acid
N	normality
NAA	1-Naphthaleneacetic acid
NaOCl	sodium hypochlorite (Chlorox)
NaOH	sodium hydroxide
O	olea
OM	olive medium
OM1	OM added with 2mg/l of BAP
OM2	OM added with 3mg/l of BAP
OM3	OM added with 4mg/l of BAP
OMmod	modified olive medium
PbHCl	Propamocarb-HCl

pH	potentiael of hydrogen
ppm	part per million
PUFA	polyunsaturated fatty acid
PV	peroxide value
%	percentage
QNF1L	quantity needed for 1 litre
SD	Standard Deviation
Sec	second
SFA	saturated fatty acid
Subsp	subspecie
TDZ	thidiazuron
UV	ultra violet
Var	variety
W/m²	watts per square meter
WPM	Woody Plant Medium
WPM1	WPM added with 2mg/l of BAP
WPM2	WPM added with 3mg/l of BAP
WPM3	WPM added with 4mg/l of BAP

1. Introduction and Problematic.

The olive plays in most Mediterranean countries a socio-economic role. It constitutes the main species of fruit, both in the number of grown trees and oil production (Brhadda *et al*., 2003). The ancient olive varieties, estimated at 2000 in the Mediterranean basin, are gradually disappearing due to the abandonment of olive orchards and this is due to their low fertility, urbanization and replacement by modern cultivars (Bartolini *et al*., 1998). Olives can tolerate moderate unfavorable environmental conditions such as drought and high temperatures during summer, and have good adaptation to different type of soils (Ruggini *et al*., 2000).

Currently, the newly introduced varieties of olive trees in Lebanon, as in the rest of the world, have significantly reduced the benefit of few varieties of high reputation. This implies the introduction of potential cultivars that are neither interesting nor adapted to local biodiversity. To limit this phenomenon of "genetic erosion", genetic selection must be conducted in the oldest plots, aged over 40 years minimum; strains whose characteristics match the expectations of growers (Lavee, 1990) must be multiplied *in vitro* and *in vivo* for genetic resource conservation and mass-production of a virus-free material (Santos *et al.*, 2003).

In order to reduce the loss of genetic authenticity of Lebanese varieties and to preserve the local genetic resources of olive (*Olea europea* L.) in Lebanon and rejuvenate it, this work aimed the conservation of the eldest trees in Lebanon from Bchealehs region, known for its tolerance to cold and good productivity, by establishing *in vitro* and *in vivo* techniques.

Grafting and cuttings are the most traditional methods used for propagation of olive trees (Zuccherelli and Zuccherelli, 2003). These methods have several disadvantages: grafting by infected material may cause the viral outbreak and spread of viruses such as Olive Latent Virus 1 and 2 (OLV-1 and OLV-2), Olive Leaf Yellowing associated Virus (OLYaV) in all grafted plants (Abdullah *et al*., 2005) especially that viral infection in the Mediterranean basin has reached in some orchards high rates that exceeded sometimes 64.5% (Saponari *et al*., 2003). Concerning cuttings, the difficulty and differences in rooting potential of varieties, presents a limitation for the rejuvenation of the olive orchards.

Other methods such suckers and ovules give a limited number of plants; while the seed propagation doesn't constitute a desirable method due to the segregation; add on that, the issued seedlings require longer time to bearing (Khattak *et al*., 2001).

The propagation by leafy cuttings under mist has become the most acceptable method throughout the world (Hartmann *et al*., 2002); that's why, development of this culture depends on the development of techniques that produce in mass quality plants, thus, allowing dissemination of selected clones and genotype performance (Abousalim *et al*., 2004). It is to note that the majority of Lebanese nurseries are selling in the market non certified material that could cause, when infected, the diffusion of viruses in Lebanese orchards.

For this and in order to maintain the genetic integrity of clones, the *in vitro* culture can overcome the difficulties of traditional methods with a conventional propagation protocol; cultivation of microcuttings and the stimulation of axillary buds and their proliferation is the most commonly used in micropropagation of woody plants (Walali, 1993); to preserve the endangered olive cultivar and to ensure homogeneous virus-free material, it would be necessary to produce olive seedlings *in vitro*.

Micropropagation and regeneration of olive plantlets *in vitro* have five stages: materials preparation, culture establishment, *in vitro* initiation, regeneration and acclimatization of plantlets (Walala and Abousalim, 1993).

First, the production of olive plantlets in tissue culture is limited by the fungal and bacterial contamination of plant material. This constitutes the major problem during the initiation phase of the olive shoots in the laboratory (Zacchini and De Agazio, 2004). This problem, due to the none spraying of plots (the high production cost), can be circumvented by the application of different fungal treatments before its introduction *in vitro*. Second, the *in vitro* propagation of olive is limited due to the poor growth, lateral bud outgrowth, variable rooting ability and acclimatization of the explants (Roussos and Pontikis, 2001). Add on that, in *Olea europea* L. the multiplication efficiency *in vitro* is dependent on culture medium, growth regulator and genotype (Ansar *et al*., 2009).

The material, consisting mainly of axillary buds, will be introduced *in vitro* on different media based on MS (Murashige and Skoog, 1962), OM (Ruggini, 1984), WPM (Lloyd and Mc Cown, 1981) added with various growth regulators (auxin and cytokinin at various concentrations) in order to study the effects of different concentrations of hormones on the plant material proliferation (Abousalim *et al*., 2004).

Concerning the *in vivo* olive culture, cuttings introduced in the nursery and treated with IBA gave a successful rooting, but poor rooted cultivars may not respond to exogenous IBA (Nahlawi *et al*., 1975). However, NAA showed more effectiveness than IBA in some plants that respond unsatisfactorily to IBA (Hartman *et al*., 2002); that's why, the Bchealehs cuttings will be treated by a talc formulation of NAA (0.2%).

Due to their importance, these techniques when succeeded, could be applied to a higher number of cultivars covering the whole country, in order to study factors influencing the regeneration capacity *in vitro* especially the altitude (humidity, temperature) and the type of soil; and to compare the rate of the olive infection (fungus and bacteria) between the seasons and then to attempt the production of plants free from viruses using different techniques such as shoot tip culture and/or somatic embryogenesis (developed from internodes, leaves, buds).

In Lebanon, the production of olives ranges between 40 and 185 thousand tons per year (Ministry of Agriculture, 2004) and the cultivated area occupies 57 thousand hectares. This culture, which occupies a large area that corresponds to 40% of Lebanese agricultural plots, covers several regions from the north to the south of the country (Koura, Akkar, Zgharta, Batroun, Chouf, Choueifat, Hasbaya, Marjayoun). On the Lebanese territory, *Olea europea* L. is grown from coast to an altitude of 1000m.

Even though the local production of olive oil only covers 36% of the country's internal needs in vegetable oils (Mouawad, 2005), this culture interfere and play an important role because it provides an extra income for almost 20% of Lebanese families.

Olive oil represents a typical lipid source of the Mediterranean diet and its consumption has been associated with a low incidence of cardiovascular diseases, neurological disorders and antioxidant properties (Vekiari *et al*., 2010). Fatty composition of olive oil is affected by several agronomical factors such cultivar, fruit ripeness, crop yield, growing medium (Beltran *et al*., 2004), environmental factors, harvesting methods and extraction techniques (Vekiari *et al*., 2010). During ripening, the content of oleic acid remains constant or shows a slight increase, while the palmitic acid decreases and linoleic acid increases. The oleic/linoleic ratio is responsible for oxidative stability as well as the natural antioxidant content (Beltran *et al*., 2004).

This work, that consisted on a technical development of a protocol for the micropropagation of Millenium trees of Bchealeh, was carried and maintained *in vitro* in the laboratory of Plant Biotechnology at the Holy Spirit University; while the production of rooted cuttings was done in the nurseries of Robinson Group at Mastita-Lebanon. Concerning the oil, it was extracted and tested in the food technology laboratories at the Holy Spirit Univeristy.

Before the introduction of the plant material, series of treatments were applied in order to minimize the infections of the newly developed explants. Culture initiation, multiplication, rooting and acclimatization of the issued material on different medias (MS, OM and WPM) added with different concentrations of cytokinins (BAP at 2, 3 and 4mg/L), and auxins (NAA and AIA at 2mg/l BAP) were tested *in vitro*.

For the *in vivo* cuttings, the plant material were introduced into a greenhouse on a mix of peat moss and potting soil during the seasons of autumn, spring and summer under controlled conditions of temperature and humidity.

Concerning the fruit weight, it shows continuous increase from the beginning of fruit development till the fruit reaches its full weight with a reduced rate of growth in the middle of development period (Desouky *et al*., 2009).

Although the Millenium trees are very important, the evolution of the fruit size and the composition of its oil had not been studied, that's why the effect of fruit ripeness and date of

collection on the extra virgin oil are studied. Series of analytical determinations will be executed as defined by EEC Regulations and are the following: free fatty acids content, peroxide value saponification value, iodine value and fatty acid composition.

This work will be divided into three parts.

In the first part, the bibliographic review, will be cited characteristics of the olive tree (cultural and genetic), the traditional breeding methods and techniques of tissue culture that can be applied in the olive for the improvement and conservation of local varieties. The olive oil quality and its components, date of collection, extraction and conservation.

The second part, material and methods, will include different steps and techniques used in this work.

- The collection and disinfection of the plant material.
- The different phases of *in vitro* culture, initiation, multiplication, rooting and acclimatization.
- The *in vivo* culture.
- Test done on olive drupes and oil depending on the extraction date and its conservation by examining its chemical composition (fatty acids composition and variation) and some of the physicochemical properties and including weight, width, pit weight, flesh/pit ratio, moisture and oil content.

The third and last part will include results and analysis of the *in vitro* and *in vivo* different phases in finding the conditions that will improve the techniques applied during the experiment and best time for the collection of olives for the oil extraction and conservation.

2. Bibliopraphic review.

2.1. The olive tree: classification, characteristics, propagation methods, cultivation and industrial aspects.

Olive is a member of the *Oleaceae*, the family that contains the genera *Fraxinus* (ash), *Forestiera neomexicana*, *Ligustrum* (privet), *Syringa* (lilac) (Martin, 1996) and a number of shrubs such as *Forsythia*, *Jasmine* (Howard *et al.*, 1989) as well as *Olea* (olive). Commercial olives belong to the species *Olea europea* L. There are about 33 species of *Olea* found in tropical and subtropical regions of the world, but only *Olea europea* L. produces edible fruit. This tree is grown in Mediterranean climate regions for its fruit and oil (Cimato, 1999).

2.1.1. Botanical classification.

The specie *Olea europaea* has long been divided into two sub-species, *Olea europea* var. *europea* for domestic olive and *Olea europea* var. *sylvestris* for oleaster or wild olive; this subdivision has become obsolete due to the differentiation in their morphological characters and geographical distribution (Van der Vossen *et al*., 2007); various studies have shown the absence of boundary between wild and cultivated populations (Breton, 2006). The wild olive trees differ from the cultivated olives by its smaller fruit (5-12mm), length, thickness and oily mesocarp; often dense, highly branched and thorny (Van der Vossen *et al*., 2007).

There are however five other subspecies:

- *Olea laperrinei* is the endemic wild olive tree from the mountains of Sahara.
- *Olea cerasiformis* from Canary Islands (*Olea cerasiformis*) and Madeira (*Olea maderiensis*).
- *Olea gaunchica*.
- *Olea cuspidata* from South Asia (*Olea cuspidata*), Arabia (*Olea chrysophylla*), Eastern and Southern Africa (*Olea africana*) is the wild olive tree widespread in tropical areas. *Olea cuspidata* can be distinguished from European olive (*O. e. europea*) by having golden brown lower leaf surfaces and smaller fruits (6-7 mm long).
- *Olea maroccana* is the wild olive from the Moroccan Atlas (Brousse and Loussert, 1999).

Consequently, there are two large populations of olive (*Olea europea* subsp. *europea*); wild populations that have high genetic diversity and populations consisting of domestic varieties, whose polymorphism is much lower, although the number of individuals is very important (Breton, 2006). There are currently more than 2000 olive varieties listed in the world and each country focuses on cultivars which differentiate by their port, phenology, and morphology of leaves and fruits (Breton *et al.*, 2006) for economical interest (Van der Vossen *et al*., 2007).

2.1.2. Historical review.

According to archaeologists, the edible olive seems to have coexisted with humans for about 5000 to 6000 years, going back to the early Bronze Age (3150 to 1200 BC). Its origin can be traced to areas along the eastern Mediterranean Coast, where are now southern Turkey, Syria, Lebanon, Palestine, and Israel based on written tablets, olive pits, and wood fragments found in ancient tombs (Vossen, 2007) and, then extended to the west and north of the Mediterranean basin. The powerful civilizations of the eastern Mediterranean such as the Phoenicians, Greeks and Romans, have spread this culture throughout the Mediterranean Basin (Brown, 2004).

Olea europea has evolved a number of adaptive mechanisms to survive the prolonged summer-drought conditions in the Mediterranean environment that appeared progressively since 10000 years BC (Moreno *et al.*, 1996; Gucci *et al*., 1997). Archaeo-biological studies (Terral, 1997) and genetic studies of populations of wild olive and olive varieties (Besnard *et al.*, 2001) showed that domestication occurred independently in several regions of the Mediterranean and had probably done over a long period.

The wild olives *Olea chrysophylla* and *Olea europea* L. var. *oleaster* most probably yielded the domesticated form *Olea europea* L.

These wild types are known to have existed in the region of Syria about 6,000 years ago (Zohary and Spiegal-Roy, 1975). From the eastern Mediterranean, olive trees were spread west throughout the Mediterranean area and into Greece, Italy, Spain, Portugal, and France.

Archaeological studies show that olive oil was already extracted in Syria and Cyprus during the fourth millennium BC; then around 1700 BC, the technology improved and the first simple "tree presses" were known in Ugarit, north of Tyre (Lebanon) (Amouretti and Comet, 2000).

By 1200 BC, the population growth in the Mediterranean bassin led to the establishment of numerous colonies by the Phoenicians in North Africa (Carthage) and south of Spain where they brought their olive growing and developed its business. In the fourth century BC, Alexander the Great conquered the eastern Mediterranean and the Persian Empire, and commerce developed further (Breton, 2006).

During the middle Ages, olive oil continued to increase in production and importance, primarily in Spain, Italy, and Greece. The greatest expansion of olive oil production came after the 1700's, when large plantings of olives, largely relegated to the worst land, were made to supply the growing populations of cities (Vossen, 2007).

In the following centuries, new production methods have been developed to face the growing demand for oil in nutrition, lighting, medical care, sports and religious practices. The olive waited until the nineteenth century, when the apogee of rural demographics and the European colonization, to reach its maximum extension (Amouretti and Comet, 2000).

In the late 19th and 20th centuries, the development of low-cost solvent extraction techniques for seed oils and the use of other sources for light (gas and electricity) resulted in a drop in the demand for olive oil (Vossen, 2007).

2.1.3. Olive and religions.

Olive tree supports the myths of the Mediterranean cultures: Bible, Qur'an, great classical Greek texts. The Bible (Old Testament) makes many references to the olive tree, the best known is the end of the Flood, where the dove dropped by Noah after the Flood (Genesis 8 / 11) returned holding an olive branch in its beak after finding an emerged land (Breton *et al.*, 2006).

In Judaism and Christianity, olive oil is used for anointing sacred and the olive tree symbolizes peace, reconciliation, grace and sacrifice (Terral, 1997). In the Qur'an, the olive tree is a blessed tree, symbol of the universal man, and olive oil is a source of divine light to guide men (moubârakatin zaytounatin in verse 35 of the 24th chapter entitled "The Light "/ An-Nur). Olive tree, Tree of the Gods, a symbol of strength, durability and peace, always tends towards the light. It embodies a way of life (Breton *et al*., 2006). On the UN flag, the crown of olive branches around the world symbolizes the universal peace.

2.1.4. Vegetative characteristics.

The old, traditional olive production system in dry-farmed areas around the Mediterranean, range in tree spacing from 7.6–18.3m apart, giving 30–173 trees/ha with an average yield 1.1–4.5 t/ha, with a long delay before full production (15–40 years) and severe alternate bearing. The trees are almost always harvested by hand or by beating the fruit off with long poles onto nets. The trunks are so large, it is difficult and expensive to harvest the trees with mechanical shakers, where individual branches are shaken (Civantos, 2001; Rallo, 2005). Good irrigated orchards can produce 10 times the yield of the old traditional olive (European Commission, 2003).

The olive tree can grow 15-20m tall and live very long. However, in extremely windy areas or exposed to the spray, it retains a bushy shape and keeps balls compact and impenetrable, giving the appearance of a thorn bush (Ereteo, 1982). In most methods of cultivation, the trees are maintained between 3 and 7m height to allow the maintenance and harvesting.

2.1.4.1. The leaf.

Olive leaves, opposite and oval, are carried by a short petiole and are curled at the edges. The foliage is evergreen, thick and leathery (Denninger *et al*., 1993). Its leaves expand within three months, during spring and are replaced after a two-year of life period; a second growth flush occurs in autumn (Diamantoglou and Mitrakos, 1981).

Leaves have stomata on their lower surfaces only. Their capacity to undergo dehydration is limited by a high internal diffusive resistance, which is due to the dense packing of mesophyll cells (Giono *et al*., 1999). Stomata are nestled in peltate trichomes that restrict water loss and

make the olive relatively resistant to drought. Some multicellular hairs are present on leaf surfaces (Martin, 1996).

A layer of peltate scales on the abaxial leaf surface are likely to function by trapping warm moist air below the stomatal aperture and consequently reducing water loss from the plant (Fahn, 1986).

Olive leaves usually abscise in the spring when they are 2 or 3 years old; however, as with other evergreens, leaves older than 3 years are often present.

2.1.4.2. The flower and inflorescence development.

The flowers, small, white and grouped in small clusters (panicles) of 10-30, grow in the axils of leaves in early spring on old stems of two years; most trees are self-fertile (Brousse and Loussert, 1999). Usually the bud is formed on the current season's growth; buds may remain dormant for more than a year and then begin growth, forming viable inflorescences with flowers a season later than expected (Martin, 1996). Pollination is done mainly by wind and lasts one week a year. To get a good production, 5 to 10% fruit set of flowers is needed (Cimato, 1999).

Two types of flowers are present each season: perfect flowers, containing stamen and pistil; and staminate flowers, containing aborted pistils and functional stamens. The proportion of perfect and staminate flowers varies with inflorescence, cultivar, and year (Martin, 1996).

The perfect flower is evidenced by its large pistil, which nearly fills the space within the floral tube. The pistil is green when immature and deep green when open at full bloom. Staminate flower pistils are tiny, barely rising above the floral tube base. The style is small and brown, greenish white, or white, and the stigma is large and plumose as it is in a functioning pistil (Martin, 1996).

2.1.4.3. The fruit.

The olive tree has a strong biennial cycle. The heavy load of fruit inhibits shoot growth that is necessary for the formation of branches that will bear fruits the following year and vice versa (Van der Vossen *et al*., 2007).

The fruit is a drupe with a skin covered with a waxy material impervious to water. The olive has a fleshy pulp rich in fat stored during lipogenesis from late August until veraison. First green, it becomes black at full maturity (Denninger *et al*., 1993). The olive fruit consists of carpel, and the wall of the ovary has both fleshy and dry portions.

The skin (exocarp) is free of hairs and contains stomata. The flesh (mesocarp) is the tissue eaten, and the pit (endocarp) encloses the seed. Fruit shape, size and pit size and surface morphology vary greatly among cultivars. The endocarp enlarges to full size and hardens by 6 weeks after full bloom. The mesocarp and exocarp continue their gradual growth (Martin, 1996).

The thick-walled stone consists of an envelope which is sclerotized during summer from late July and has an almond with two ovaries, one of which is usually sterile and non-functional (Barranco *et al.*, 1992) : the seed (rarely two) produces an embryo, which will give new olive if conditions are favorable.

2.1.5. Climatic requirements and cultivation aspects.

Olea europea has evolved a number of adaptive mechanisms to survive the prolonged summer-drought conditions in the Mediterranean environment, which affect water status and CO2 assimilation (Moreno *et al.*, 1996; Gucci *et al*., 1997). The olive is very hardy and indifferent to the soil. It fears humidity, and do not suffer from exaggerated high winds (Terral, 1997).

2.1.5.1. Temperature.

As the olive tree cannot withstand temperatures below -10°C isotherm that delineates its cultivation area in latitude (usually 25°-45°) and altitude above sea level (maximum 900m) (Miouline, 2002). This tree withstands -10°C in winter dormancy, but at 0°C, the damage can be very important on flowering (Amouretti and Comet, 2000), although a marked winter is necessary to induce the production of flowers and therefore olives. However, at 35-38°C vegetative growth stops and at 40°C and higher burns damage the leaves and can bring down the fruit, especially if irrigation is insufficient (Bush and Loussert, 1999). Flower initiation requires, for most cultivars, a vernalization period of 6 to 11 weeks at a temperature of 9°C ending 40-60 days before anthesis. The optimum temperature for shoot growth and flowering are 18-22°C degrees (Van der Vossen *et al*., 2007).

The hot winds during the bloom, fog and high humidity, hail and late frosts are factors unfavorable to flowering and fruiting.

2.1.5.2. The soil.

The olive tree is most often grown in poor soils and dry, but its use in rich (California) or watered (Spain and Oran) lands gives remarkable results (COI 1, 2006). The soil should be of light texture, less than 20% clay, well drained and a minimum depth of 1.5m. Olive trees grow well on very poor soil unless it's waterlogged, saline or too alkaline (pH> 8.5) (Van de Vossen *et al*., 2007).

2.1.5.3. The pluviometry.

The olive fears excessive watering (intake of 300-400 liters of water, 1-2 times in July and August, and only the first year after planting) (Devaux, 1993). With a well distributed rain (600mm) over the year, the olive tree grows and produces normally.

Between 450 and 600 mm/year, production is possible if the holding capacity of water in the soil is sufficient or tree density is lower; with a rainfall of less than 200 mm / year, the olive may be economically inefficient (Ereteo, 1982).

Some olive cultivars, such as those grown in Crete, southern Greece, Egypt, Israel, and Tunisia, bloom and fruit heavily with very little winter chilling; whereas those originating in Italy, Spain, and California require substantial chilling for good fruiting (Martin, 1996).

2.1.5.4. Cultural practices

The production of olives occurs naturally in alternate years in the absence of pruning, and production begins slowly, gradually and becomes permanently: during the first seven years, the tree is unproductive; this time can double in case of drought; up to 35 years, the tree grows and is experiencing a gradual increase in production; between 35 and 150 years, the olive tree reaches full maturity and maximum output. Beyond 150 years, it ages and its yields become random (Denninger *et al*., 1993).

2.1.6. The olive oil production

2.1.6.1. World production

The oil production is concentrated in the Mediterranean countries (98% of world production). The olive occupies a very important part in the agricultural economy of Mediterranean countries and the trend of global consumption is increasing yearly (UNCTAD, 2005).

Although the area of olive groves has diminished over the twentieth century, gain in productivity in the cultivation of olives and oil extraction have led to fivefold increase in world production of olive oil between 1903 and 1998 (FAO, 2005). World production of olive oil rose from 1.453,000 tons in 1990 to 2,820,000 tons in 2006 (IOC 1, 2006) while at the same time, production of table olives rose from 950,000 tons to 1,832,500 tons (IOC 2, 2006) (Figure 1).

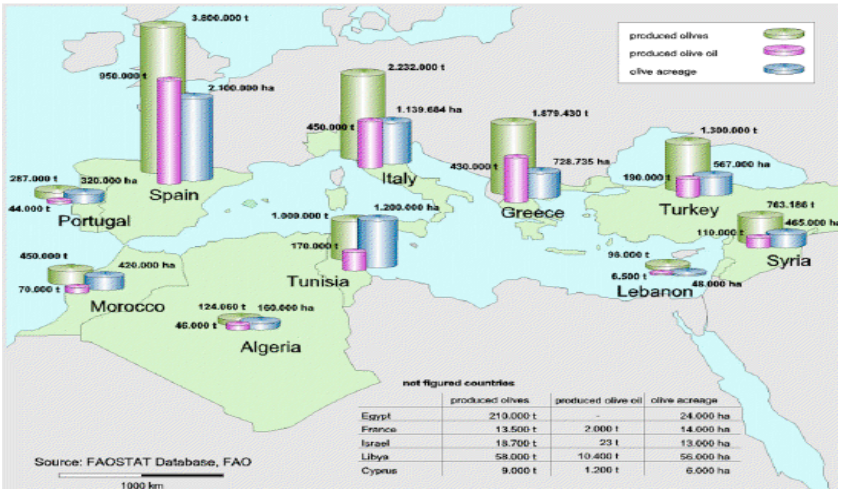


Figure 1. Mediterranean distribution of olive production (FAO, 2005).

The world has 9.4 million ha of olive orchards, producing 1.5 million tons of table olives and 16 million tons of olives that are processed into 2.56 million tons of oil. The EU provides 80% and consumes 71% of world production of olive oil (Ollivier, 2003).

Spain has about one-quarter of the world's acreage, with 2.42 million ha of olive trees under cultivation and 36% of the oil production (1,119,100 tons/year), which ranks it as the top producer (Figure 2). During the season of 2001–2002, Spain produced 49% of the world's olive oil as a result of a very high yield and poor crops in many other countries.

Italy is ranked second, with 1.43 million ha and 25% of the world's olive oil production with 520,000 t/year. Greece is third, with 17% (400,000 t/year) of the world's olive oil production, and is fourth in world acreage with 1.03 million ha of olive trees. Together, the big three produce 78% of the world's olive oil (FAO, 2005; IOOC, 2003a, 2004) (Table 1).

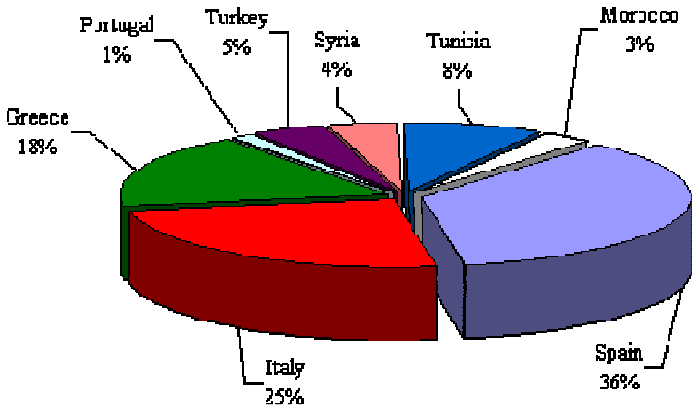


Figure 2. Main producing countries in 2005 (FAO, 2005).

2.1.6.2. Lebanese production.

In Lebanon, olive oil production is around 76.5 thousand tons of olives per year, with an average of only 0.8% of world production in good harvest years (Ministry of Agriculture, 2004). The cultivated area occupies 55,000 hectares and covers several Lebanese regions stretching from north to south (Koura, Akkar, Zgharta, Batroun, Chouf, Choueifat, Hasbaya, Marjayoun). Yet, the local production of olive oil only covers 18% of the country's internal needs in vegetables oils (Mouawad, 2005). More than 70% of the total olive area is destined to olive oil production and the remaining is consumed as table olives (FAO, 2007).

Table 1. World's leading producers of olive oil (FAO, 2005).

	Spain	Italy	Greece	Turkey	Syria	Lebanon
Percentage of world production (%)	36	25	17	5	4	0.19
Oil production (thousand tons)	1.119	520	400	180	145	6.5
World ranking	1	2	3	5	6	13

2.1.7. Industrial relevance.

Until the nineteenth century, olive oil lamp was widely used to soften and lubricate the tissues and textile fibers in spinning, and as one of the most powerful natural non-drying lubricants for mechanics, since it has excellent viscosity; it evaporates and turn into a gummy residue and sticky very slowly (Amouretti and Comet, 2000).

Oil production for the industry is small (50,000 tons in 1999, representing 2% of world production of olive oil) and concentrated in Syria, Tunisia and Turkey. It is used locally and is barely exported (6,000 tons exported in 1999) (UNCTAD, 2005).

In Italy and Spain, there are factories that produce electricity using the pomace as fuel (the solid residues from the manufacture of olive oil). In some Lebanese agricultural enterprises, boilers (manufactured in Syria) that run on olive pomace were recently introduced in nurseries in order to reduce the cost of production given the continued increase in global fuel prices. These will progressively replace the boilers that use fuel oil for heating nurseries. The pomace could be also used to feed livestock (CIHEAM 3, 2002) or for the production of olive pomace oil in order to reduce environmental pollution.

2.2. Propagation mode.

Plant propagation consists on the application of specific biological principals and particular techniques for the multiplication of plants. The plant obtained in the process should be identical or as similar as possible to the plants from which they derived (Fabbri *et al.*, 2004).

The olive tree can be propagated by two types of processes: the traditional (wood cuttings, and division of strain release "Sprouts" and grafting onto wild olive) and intensive methods (grafting, cutting treated with hormone). The intensive process including all stages is done in greenhouse equipped with fogging irrigation system (Denninger *et al*., 1993).

The creation of new high yielding varieties can be arranged by controlling the pollination process and following the qualitative and quantitative performances of a large number of off spring (Cimato, 1999).

Shoots and branches are excellent propagation material when the proper techniques are applied. The olive tree is very efficient at differentiating new meristems at the collar where the transition from trunk to root system takes places. This explains the relative ease in propagating the olive from larges plant parts, which enabled ancient growers to asexually propagate this tree (Fabbri *et al.*, 2004).

2.2.1. Seedling.

All current varieties results from domestication and sexual reproduction of olive under the selective pressure of human use. A plant issued from the germination of a kernel has its own characteristics and unique, even if derived from selfing. An olive pit from a cultivated variety does not necessarily give an interesting or same variety; and therefore, not necessarily accesses to the rank of variety (Breton, 2006), even if flowers leading to this nucleus have been fertilized by pollen from the same variety (Devaux, 1993).

The seed, horticulturally mature by October, is harvested when ripe but before they turn black; then they are stratified to achieve maximum germination (Lagarda *et al.*, 1983a); once the fruit is physiologically mature, in January, seed germination will be reduced (Martin, 1996).

Add on that, planting an olive pit and wait the development of the tree is a risky method because the nuclei are very strong and must be split or weakened in order to germinate (Franclet, 1979). That's why, pre germination treatments are designed to overcome the dormancy of the seedcoat and embryo. Mechanical or chemical scarification is used to treat mechanical dormancy. Pits may be soaked in concentrated sulfuric acid to soften the endocarp. The acid bath is then followed by 1 to 2 hours of rinsing in water (Crisosto and Sutter, 1985). The pits can be planted directly after the endocarp treatments.

The most successful of these treatments on a commercial scale is stratification. Pits are scarified and then soaked in water at room temperature for 24 hours. The pits, mixed with moist sand, are placed in the dark in a controlled environment at a constant room temperature (15ºC) for 30 days. Stratification is thought to reduce abscisic acid that inhibits germination, within the embryo or seed coat (Martin, 1996).

To preserve the genotype of the selected varieties for their qualities and cultivate olive exactly identical to the original, growers prefer propagating asexually (although clonal mutations can occur) by cuttings, grafting, or by "Sprout" (piece of stem) (Pans, 1960). Thus, the best performing varieties for the production of olives and oil have increased, while the least efficient varieties are neglected and forgotten (Breton, 2006).

2.2.2. Softwood cuttings.

The most used method for the multiplication of the olive tree is rooting softwood cuttings taken from one-year shoot (10-12cm in length and 4-5 buds) (Van der Vossen *et al.*, 2007). Propagation by shoots gives fast and excellent results when cuttings of sufficient size are chosen. This technique has the advantage to reproduce with certainty, all year around the same variety, without grafting (Barranco *et al.*, 1992); it is important when making softwood cuttings to obtain the proper type of cutting material from the stock plant (Laubscher, 1999).

It is practiced in the nursery to produce seedlings from mother feet, first identified for their production qualities and their health status (Pans, 1960). This technique uses shoots developed in the same year, or 1-year-old shoots which have not produced fruit (Fabbri *et al*., 2004). This method reduces transpiration and leaf temperature and increases relative humidity, enabling cuttings to remain turgid throughout root induction (Sibbett *et al.*, 1994).

According to Hartmann *et al.* (1990), for extremely fast-growing, soft tender shoots are not desirable, as they are likely to wilt and dry out before rooting. This is done in relation to the production cycle of self rooted plants which generally coincides with two annual peaks points of concentration of rooting compounds within tissues, i.e. April and September-October in Central Italy.

Shoots can be divided into 10-15cm long pieces of 4-6mm in diameter with 4 to 6 nodes. The 4 distal end leaves are retained while the 2 to 3 basal nodes are left without leaves. The basic requirements of propagation mixtures should be a well-drained, open mixture with the capacity to retain moisture (Gardiner, 1988). The basal cut must be made just under a node. Generally, new roots do not grow from internode tissues; they usually develop within node tissues. Once the cuttings are prepared, basal treatment with a rooting hormone must be carried out within 20 to 40

minutes. Including high proportions of peat moss in the rooting medium considerably increases the mixture's water-holding capacity which can cause deterioration of the roots (Denisen, 1979). The introduction of the misting technique for shoot rooting has provided a valid alternative to the old techniques used (Fabbri *et al*., 2004).

Small tunnel, that are plastic covered, can be used for rooting of cuttings and for raising seedlings. Temperature, humidity and light play an important role in the initiation of the culture. The optimum temperature for rooting medium should be 18- 21°C while the optimum ambient temperature could range from 21 to 26°C. Concerning the humidity, its primordial for cuttings to keep the environment close to saturation (>80%) until the development of roots. The recommended lighting varies between 5000 to 10000 lux. Light acts by its intensity, length and its quality on the photosynthetic activity of the leaves and the synthesis of rooting promoters.

Establishing and maintaining stable uniform environmental conditions are essential for rooting of the cuttings. Hartmann *et al.* (1990) found that mist units, warm benches and cold frames enhance quicker rooting. More uniform results are likely to be obtained under these conditions, because the absorption of the chemicals by the cuttings is not influenced as much by surrounding conditions as by the rooting powder (Lamb *et al*., 1975). Extreme fluctuations of these conditions can retard or inhibit rooting (Hartmann *et al*., 1990).

2.2.3. Wounding.

The bases of stem cuttings are wounded to release chemicals, in order to stimulate root production. It consists of making thin longitudinal incisions at the base of cuttings with a sharp knife, before auxin treatment (Fabbri *et al*., 2004). Wounding exposes more young cells, increases the absorption rate of water, allows easier penetration of rooting hormones and removes barriers such as thick cell walls (Laubscher, 1999) which leads to a greater callus and adventitious root production at the edges of the incisions. Hartmann *et al.*, (1990) suggested that root promoting treatments will not only increase the percentage of rooting of difficult rooting plants, but will also be beneficial with easily rooting plants. Rooting auxins can also increase the number and the quality of roots and promote uniformity in rooting.

In case of talc formulations, auxin is dispersed in the inert talcum powder. The cuttings are dipped in the powder and lightly tapped to remove excess powder. In order to avoid brushing off the powder during insertion of cuttings in the rooting medium, a stick can be used to make a hole in the medium before cutting is inserted (Fabbri *et al*., 2004).

Rooting promoting hormones

Auxins are root-promoting chemical agents. Indole Butyric acid (IBA) and Naphthalene Acetic acid (NAA) are two synthetic growth regulators that have been found most reliable to stimulate adventitious rooting in cuttings (Hartmann *et al*., 1990). Combinations of these chemicals give improved results (Lamb *et al.*, 1975) and have shown to be stronger root promoters, light and temperature stable, and more resistant to microbial decomposition. NAA is stronger than IBA in terms of stimulation of olive adventitious rooting but uniformity of results is difficult to obtain (Fabbri *et al*., 2004). As the majority of woody plants, the most widely used rooting hormone for olive cuttings is Indole Butyric Acid (IBA) and the most common method used for application is to quickly dip the base of cuttings for 3-5 seconds in a 3000 ppm IBA solution (FAO, 2011)

The effectiveness of auxin on root initiation depends on its critical level of concentration, whereas those above that level can inhibit root growth and bud development (Janick, 1986).

Concerning the effective concentrations of IBA, the range of 2000 to 4000 ppm in hydro-alcoholic solutions give the best result with reference to the period of cutting collection; 2000 ppm when natural rooting hormone levels are high (cuttings collected from vigorous vegetative shoots of the stock plant), 4000 ppm when natural rooting hormone levels are low (cuttings collected during the rest period or during bloom) (Fabbri *et al*., 2004). With respect to NAA, treatments with this growth regulator are generally in range of 1000-2000 ppm.

Narrow leaved evergreen cuttings ordinarily were best taken between late autumn and late winter. Cuttings will respond better from early to mid-summer plantings under a relative humidity of 70-80% provided by inter mist spray and bottom heat (Hartmann *et al.*, 1990).

2.2.4. Woody Branches.

Used by the Phonenicians, Romans and Arabs, this method makes use of the possibility of producing shoots and roots from 4 to 5 years old branches. Cuttings, 20 to 50cm long with a diameter of 5 to 10 mm, are obtained from branches during the cold season (autumn-winter) (Fabbri *et al*., 2004). Unfortunately, this propagation requires the use of large quantities of wood that it is often difficult to find on the pruning from orchards; that's why it couldn't be applied on commercial scale (Denninger *et al*., 1993). The new shoots and roots are produced from the living tissues that are in the cortex when the piece of wood is buried for a few centimeters (Fabbri *et al*., 2004).

The plant material used for the production of cuttings differs in its mode of planting nursery (Denninger *et al*., 1993). The different types of cuttings (Fig.3):

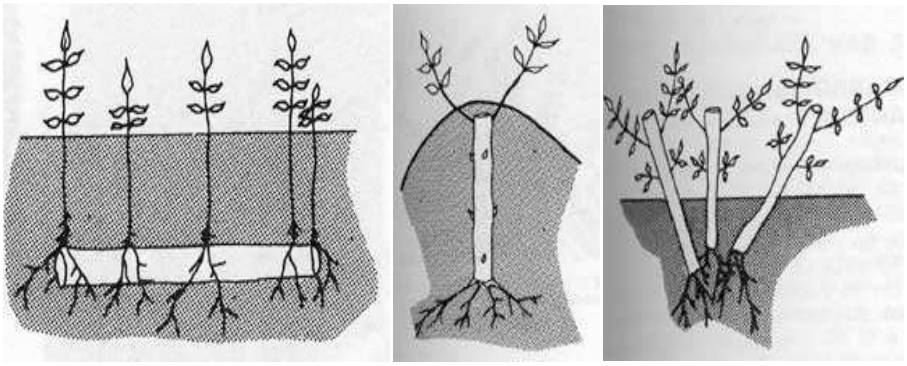


Figure 3. Horizontal, vertical and, stake cuttings.

2.2.5. Sprouts.

In nature, when Olive gets old, it produces sprouts from its stump, and never actually dies of old age. The new tree that replaces it is not another olive tree, but a second self, a new expression of the same genotype (Barranco *et al.*, 1992).

At the base of mature trees (30 years) are born small shoots called sprouts. These are projections that could be taken from olive strains and used for direct placement or for the establishment of nurseries. Sometimes, the sucker can be erected near the mother plant without being detached. When it is sufficiently developed, it will replace the olive mother who will be gradually eliminated (Pans, 1960). These pieces of stem (1cm in diameter), who emit roots quickly, can be chosen with or without a bit of bark that facilitate the nutrition of young shoots during the early

years (Franclet, 1979). After at least two years, it is recommended to replant in the ground and on the same plot as the levy for the "variety" has adapted to its environment.
This method is particularly useful for planting in land and in a very dry climate (Pans, 1960) but cannot be used at nursery level because it is slow and costly. Multiplication by sprouts does not always produce the desired variety; for this, grafting will be a must (Cimato, 1999).

The layering in clump is better young discharges that develop on the mother plant so as to promote the emergence of young roots. After withdrawal of layers, releases are rooted in established orchards. Releases of small diameter, relatively sensitive to drought, will be eliminated (Denninger *et al*., 1993).

2.2.6. Grafting.

The proliferation of an olive could be also done by grafting, except in special cases (cuttings and souquets) (Miouline, 2002).

Seedlings and rooted cuttings, issued from softwood cuttings, may be used one year later as rootstocks to propagate cultivars that are difficult to root (Sibbett *et al.*, 1994).

The following techniques could be used: for seedlings and sprouts of a different variety, the cleft grafting or patch may be used (Pans, 1960); for older trees, it should adopt the veneer grafting or crown-grafting under bark (Denninger *et al*., 1993). These operations, carried out preferably in the spring, carried on large branches of carpentry. The veneer grafting, highly recommended, requires a very tight ligation and girdling of the branch carpenter above the graft.

2.2.7. Ovuli.

Ovuli are proteburances composed of meristematic tissue that appear at the level of the collar. Ovuli can be induced to form shoots and roots by removing them from the mother tree (Sibbett *et al.*, 1994).

It is most often found at the base of the trunk where the root structure joins the trunk producing torsions in the vessels and thus slowing sap circulation. This is due to a hyper-nutrition of

cambium cells, which proliferate and produce the extroflection of tissues, particularly parenchyma tissues that constitute most of the ovule (Fabbri *et al*., 2004).

Ovules are generally taken from the collar which is particularly developed in old olive trees (Baldini 1986; Hartmann *et al*., 1990). The ovuli, when removed, need a great care and must be disinfected and protected. The bark of the ovule must be smooth and light in color with slight wrinkling which reveals the presence of latent buds, and the wood must have a healthy appearance (Fabbri *et al*., 2004) but the damage done to the mother plant limits their use for propagation (Sibbett *et al.*, 1994).

The annual production of olive trees in the main olive-growing countries of the world is around 40 million, with 32 million in the Mediterranean basin and 8 million in the rest of the world (IOOC, 2000). The mist technique is the most common propagation method with 28 million trees followed by grafting (7 million) and 5 million by traditional techniques (ovules, cutting from branches) (Fabbri *et al*., 2004).

2.3. Biodiversity and biotechnology.

2.3.1. Biodiversity.

The number of landraces of olive, estimated at 2000 in the Mediterranean Basin, is gradually disappearing due to the abandonment of groves with low olive fertility, urbanization or replacement by modern cultivars (Bartolini *et al.*, 1998). This variability can be reduced once the problem of synonymy and homonymy from the environment and pathogens is solved. There is a great need for genetic certification of olive and new biotechnology techniques that can save the genotypes with particular characteristics by using genetically identification, protecting them from viruses and preserving them through unconventional techniques (Ruggini, 2002). Programs to collect and preserve these valuable genetic resources are ongoing, with support from the International Olive Council (IOC) and the European Union (Van der Vossen *et al.*, 2007).

Currently, the olive trees varieties grown in Lebanon, as in the rest of the world has significantly reduced in favor of some varieties of high reputation. To limit this phenomenon of "genetic erosion", genetic screening must be done on strains whose characteristics match the expectations of growers by identifying in older plots, the trees aged 40 years or more, (Lavee, 1990) to

multiply it *in vitro* for the conservation of the genetic resource and mass production of virus-free material (Santos *et al*., 2003).

Material from *in vitro* culture will be tested concerning the most prevalent virus, Olive Latent Ring Spot Virus (OLRSV) and Olive Leaf Yellowing-associated Virus (OLYaV) (Saponara *et al.*, 2003), then planted in order to study its agronomic characteristics in orchards. The trees of the best strains are then multiplied and offered to growers.

2.3.2. Biotechnology.

Biotechnology contributes significantly to the genetic improvement of olive (Ruggini, 2002), thus it is necessary to use *in vitro* culture. This culture depends on the totipotency of plant cells defined as the ability of any organ (shoot, root, leaf, anther, pollen grain) or fragment (microcuttings, bud tip) to be separately cultured *in vitro* on a synthetic nutrient medium under aseptic conditions (absence of bacteria and fungi) using various techniques (Walali and Abousalim, 2006).

In vitro culture objectives are numerous and varied: differentiation and production of identical plants (micropropagation), healing (meristem culture and grafting), improvement (anther culture) and selection of mutants (INRA, 1997).

2.3.2.1. Micropropagation.

The plants can be reproduced in two ways: asexually (vegetative propagation) and sexually. The characteristic of vegetative propagation is that the issued plants are genetically identical to the mother plant: this is called cloning (CIDES, 1999).

The micropropagation *in vitro*, which derived from this natural phenomenon, is applied in horticulture for cloning. Sterile explants are introduced on an artificial medium and under controlled conditions (INRA, 2001). It allows the mass production of cultivars and selections of interesting explants after several subcultures (Ruggini, 2006) and, the multiplication of species difficult to reproduce by conventional ways. The plants obtained are multiplied to infinity.

Micropropagation allows production organizing regardless seasons and, reduces the cost of production (Cevie, 1997). Regarding fruit species, it is possible to overcome the rootstocks problem when applying *in vitro* multiplication. Thus, the obtained shrubs do not show the problem of scion rejection (Techniver, 2007). *In vitro* propagation of olive explants has not yet passed the experimental stage among other species, due to the wide variation of success rates between cultivars (Van der Vossen *et al*., 2007).

2.3.2.1.1. Conditioning phase of mother plants.

This step contributes significantly to the success of later phases. It refers to the preparation of mother plant which should be devoid of mineral deficiencies and therefore full turgescence, i.e. without any water stress (CIDES, 1999). In almost all cases, the seedlings should not be cultivated during its period of dormancy. Infested plants, with insects or fungal diseases, may provide significant challenges in a culture chamber (INRA, 1997). For example, thrips eggs that lodge in the interstices of buds withstand sterilization apical surface (Van der Vossen *et al*., 2007). Subsequently, favorable conditions will lead to the development the displacement of larvae inside the tubes or jars.

Concerning fungal diseases and in order to reduce the risk of contamination in olives, stock plants of the variety 'Picholine marocaine' have been treated with cryptanol, a fungicide, 15 days before the introduction of explants *in vitro* (Brhadda *et al*, 2003). Thus for a successful initiation, it's necessary to prepare the mother plants.

2.3.2.1.2. Establishment or initiation phase.

The establishment phase is the most critical phase during which crops must overcome two problems: the fungal and bacterial contamination (CIDES, 1999), and the proliferation of axillaries buds of the introduced explants in which auxin/cytokinin balance plays a crucial role (Zuccherrelli and Zuccherrelli, 2003). The buds are introduced and cultured in a media containing various components to ensure its proliferation.

The most used medias among the olive, are based on Murashige and Skoog (1962) (Chaari-Rkhis *et al*., 2006) and the olive media of Ruggini (1984) (Brhadda *et al*. 2003). These media are often

fortified with vitamins and growth regulators (Sakunasingh *et al*., 2004). The introduced explant may be an axillary or an apical bud (Zryd, 1988). The *in vitro* olive culture requires a photoperiod of 16h/d with a light intensity of 4000 lux (Abousalim *et al*., 2004). The duration of this phase depends on the genotype and the sampling time of plant material (Techniver, 2007).

2.3.2.1.3. Multiplication phase.

This stage consists on transplanting shoots obtained from the initiation stage. Increasing the number of plants is sought during this phase. The multiplication rate, a factor of 4 to 5 at each cycle in woody plants (CIDES, 1999), is the major economic criterion for commercial propagation (Zryd, 1988).

The used medium is often identical to the first, although some minor differences could take place in the hormonal balance (equilibrium auxin-cytokinin) (Zuccherrelli and Zuccherrelli, 2003). At the multiplication phase, cytokinins are generally found in higher concentration in the medium then auxins. This is due to a physiological point of view by the fact that cytokinins oppose the apical dominance stimulating thus the growth of new stems (Cevie, 1997). If the species under cultivation is slow growing, slow development can be observed in the tubes (INRA, 2001).

During this phase, several media have been tested successfully on olives giving significant results. Murashige et Skoog media (Ferreira *et al*., 2003), olive media (OM) (Abousalim *et al*., 2004) and Lloyd and Mc Cown (WPM) (Santos *et al*., 2003), added by BAP (Sakunasingh *et al*., 2004) or Zeatin at different concentrations (Abousalim *et al*., 2004), gave the best multiplication rates. The most suitable cytokinin for micropropagation of the olive tree is the Zeatin (Rugini, 1984; Mencuccini *et al.* 1995; Rokba *et al*., 2000).

2.3.2.1.4. Rooting phase.

This stage is characterized by the emergence of roots on leafy stems obtained in the multiplication phase (CIDES, 1999). Sometimes, the species have a root system more or less developed during the multiplication phase. The media varies somewhat from the previous settings. The minerals and vitamins remain generally the same. The major difference lies primarily on the hormonal balance in favor of auxins (INRA, 1997). Stems very leafy and well

equipped buds take root easily in an environment devoid of growth regulators (Augé *et al*., 1989). The young leaves and buds are natural sites of auxin; similarly to the traditional cuttings, these seedlings will root by themselves; while, some species require the addition of auxins to stimulate rooting. This auxin is often provided as IBA or NAA (Chaari-Rkhis *et al*., 2006).

In the adult olive tree, difficult rooting was mentioned by Walali (Walali and Abousalim, 1993). In the case of juvenile material, the presence of NAA at different concentrations induced rhizogenesis (Abousalim *et al*., 2005).

The difficulty of rooting *in vitro* the adult material is common to different types of wood, fruit and forest. Maximum rooting (75%) was obtained from the material multiplied on MS medium supplemented with 10mg/l IAA (Shibli *et al*., 2000). To improve its responsiveness for *in vitro* rooting, rejuvenation of the olive tree can be applied by various means may improve the rate of rooting (Franclet, 1979).

2.3.2.1.5. Acclimatization phase.

This is the last phase for the *in vitro* process. It is the gradual adaptation of seedlings in the greenhouse or in the field. After removing the agar from the base of the plants, they are transferred to a horticultural substrate composed primarily of a mixture of compost and soil (Techniver, 2007) or a mixture of vermiculite-perlite with a 3:1 ratio (Peixe *et al*., 2007). The aerial parts are then covered to keep them in around 100% relative humidity. Then, humidity is reduced progressively during the acclimation phase of plant material. The stomata of young leaves cultured *in vitro* remain constantly open due to the high risk of dehydration (CIDES, 1999). The growth of new functional leaves is necessary before the gradual removal the film.

Molecular analysis can be performed using the RAPD technique and that for the evaluation of the olive obtained by micropropagation and the somaclonal variation risks micropropagation of *in vitro* material (Leva *et al*. 2006; García-Ferriz *et al*., 2003).

2.3.2.2. Somatic embryogenesis.

Somatic embryogenesis is a powerful tool not only for the *in vitro* propagation but also for the genetic improvement of important species (such as transgenic regeneration) (Abousalim *et al*.,

2005). This technique provides a multitude of plants genetically identical to the donor plant explants from non-sexual somatic cells (Techniver, 2007). Tissues introduced *in vitro* cause, through the provision of a high dose of auxin, many cell divisions and callus development which may give rise to bipolar embryos (CIDES, 1999).

Somatic embryogenesis allows rejuvenation of tissues and access to genetic transformation. It is to note that passing by a callus stage and a prolonged culture may lead to a risk of genetic drift leading to somaclonal variants, plants whose genome is significantly different from the initial plant (Auge *et al*., 1989).

Among the olive trees, the somatic embryogenesis has been obtained from different genotypes and plants which are regenerated *in vitro* and developed successfully in the field (Abousalim *et al*., 1993). The presence of growth regulators in media plays an important role in the induction of callus and embryo development (Leva *et al*., 1995).

For the Moroccan Picholine, 40% of the material developed embryos after different stages of induction and development (Abousalim *et al*., 2005).

2.3.2.3. Technical improvement

2.3.2.3.1. Haploid plants and creation of Doubled haploid plant.

Haploid plants are issued from a male or a female sex cell without pollination. Haploid individuals are privileged material for the study of gene expression (INRA, 2001). The obtained plants that have only one set of chromosomes, naturally or artificially doubled, became fertile (Kongjika *et al*., 2000). They can be obtained by androgenesis, gynogenesis by pollination from irradiated pollen or interspecific crosses. The anther is a new biotechnology techniques used in the olive tree to the creation of new cultivars (Pintos *et al*., 2007).

Androgenetic plants

The culture of anthers or microspores from the olive tree is a useful method to produce doubled haploids from pollen (Benlhabib, 2006). It is the regeneration of whole plants from male cell:

immature pollen grains or pollen isolated by culture or by anther culture. After a spontaneous or artificial doubling by colchicines, doubled haploids are obtained (Kongjika *et al*., 2000).

This technique brings a significant gain of time, allowing to quickly creating new varieties. Thus, imbred lines are produced in a few months instead of 8 to 10 years by conventional technique of selfing (CIDES, 1999).

Obtaining pure lines is almost always necessary step in plant breeding programs (Ruggini, 2002). It is a technique used in wheat, rice, potato, tobacco, corn, and routine in oilseed rape and barley (Techniver, 2007).

The microspore culture has enabled the achievement of pro-embryos of olives for the first time. The power of embryogenic microspores was largely dependent on their stage of sampling and the morphology of floral bouquet. The newly formed pro-embryos represent the first phase of haploid formation (Bueno *et al*., 2004a, 2006).

Thus, plants obtained by this technique are completely homozygous, it can reveal interesting traits by the expression of recessive alleles usually hidden (Mohan and Häggman, 2007). The low rate of induction and regeneration of plants is a major constraint to the implementation of these methods.

Recombinants of interest can be detected and exploited such as resistance to a specific disease. For example, the resistance to *Spilocaea oleagina* was found in Israel and, the resistance to *Pseudomonas syringae* pv. *savastanoi* has been reported in Portugal (Van der Vossen *et al*.,2007).

2.3.2.3.2. Shoot tip culture.

The increasing need for new varieties for the rejuvenation of orchards has promoted the use of imports (inter and intra-regional movement of plants) of dubious origin (CIDES, 1999). This allows the introduction and spread of viral diseases that can cause adverse effects on production, quality and longevity of fruit plantations. The technical development of sanitation protocols for plant material enables the production of virus-free material (Abousalim *et al*., 2005).

Indeed 13 viruses, including the Olive Latent Ring spot Virus (OLRsV) and Olive Leaf Yellowing-associated Virus (OLYaV) have been identified (Saponari *et al*., 2003) and their symptoms were described (Abousalim *et al*., 2005; Savino *et al*. 1996; Savino and Gallitelli, 1983). The methods of struggle against these viruses borrow from multiple pathways using different techniques of regeneration *in vitro* such as the shoot tip culture and grafting.

Shoot tip, areas of intense divisions are located in the heart of buds and tips of roots and origin of leafy stem or root system (Techniver, 2007). Meristem culture is one of the most widely used techniques to produce plants free of viruses; this culture allows the rescue of endangered varieties (Augé *et al*., 1989). It concerns mainly the plants propagated vegetatively, which facilitates the transmission of the virus to new plants.

Meristem culture combined with thermotherapy (culture at high temperature) has not yet been successful to eliminate these viruses (Ruggini and Gutiérrez, 2006). In the olive, meristems (0.5-0.8mm) were taken and used to initiate the proliferation of buds in the cultivar Pantaloon (Ramzan Khan *et al*., 2002). The meristem culture of two Italian cultivars allowed obtaining a virus-free material (Ruggini and Gutiérrez, 2006). The plants obtained from meristems have increased vigor and quality of flowering and restored fruiting.

Cryopreservation of meristems applied to the olive tree has helped the recovery of 15% of the material (cv. Frantoio), after incubation at 0°C in a vitrification solution for 60 min (Benelli *et al*., 2004) and 30% in case of cv. Arbequina after soaking in liquid nitrogen (Martinez *et al*., 1999).

2.3.2.3.3. Grafting.

The olive tree is challenging to develop shoots from meristems (Ruggini, 2002). The grafting may be a method of support for meristem culture because not all genotypes are capable of developing or rejuveniler recalcitrant genotypes *in vitro* conditions. To work around this problem, Revilla *et al.* (1996) have successfully rejuvenate Arbequina cultivar after a cycle of grafting on seedlings germinated *in vitro*. Troncoso *et al.* (1999) have developed a protocol for

grafting under *in vitro* conditions and obtained from seedlings germinated *in vitro* and micrografts, 67% of naturalized plants.

2.3.2.4. Selection.

The olive varieties have emerged with the domestication when humans have sought to increase the number of trees that gave them the most satisfaction from those that had their natural environment (Breton, 2006). The improvement of the olive tree results from a long tradition of clonal selection. The crosses inter-cultivars, followed by a selection from segregated populations of plants, are relatively recent (Lavee, 1990).

The creation of new high yielding varieties can be arranged by parents chosen wisely a control of pollination and, then following the qualitative and quantitative performance of a large number of descendants (Breton, 2006). The main criteria for selection are: the fruit yield, regularity of production, cold tolerance, earliness of the first fruiting, compact growth, oil content of the mesocarp, the oil quality and resistance diseases and pests (Villmur and Dosba, 1997).

Progress in selection had been made with the application of molecular biology to the olive: use of molecular markers for cultivar identification, establishment of a linkage map and marker assisted selection (Ruggini, 2002).

2.4. Olive oil.

Olive oil is the main source of intake of fat in Mediterranean food diets. This type of diet has often been associated with better resistance to certain diseases, including cardiovascular diseases and degenerative diseases.

Numerous scientific studies were therefore interested to content nutritional olive oil in order to understand the mechanisms of action may explain these phenomena (Veillet, 2010). The main product of the olive tree is the edible oil extracted from the mesocarp (pulp) of the fruit. It is particularly appreciated for its flavor and its specific benefits to health because of its high concentration of monounsaturated fatty acids and polyphenolic antioxidants.

More than two thousand cultivars are known, they are classified into three groups:

- Cultivars for oil extraction, such as "Frantoio" in Italy and "Spanish Arbequina".
- Cultivars for production of table olives, such as "Chalkidi" in Greece and "Oliva di Cerignola" in Italy.
- Cultivars having two objectives, oil extraction and consumption of fruit, such as "Moroccan Picholine" and "Dan" in Syria.

The content of virgin oils depends on the variety of olive, the degree of maturity of olives (the amount decreases with over-ripening of olives), the level of infestation by the olive fly *Dacus olea*, climate and soil quality. The virgin olive oil is one of the few vegetable oils that are marketed and consumed without any refining and retains its full content of secondary compounds. The development of the International Olive Oil Council was in part a response to widespread fraud in the olive oil trade surrounding the sale of olive pomace oil, mislabeled as extra virgin olive oil (EC, 2001; IOOC; 2003b).

The main component of olive leaves is oleuropein, with other phenolic compounds, are responsible for the intense bitterness of the olives, the blackening of the fruit and inhibition of micro organisms development during processing. The early stages of fruit processing significantly reduce the bitterness of the olives (Van der Vossen *et al*., 2007).

2.4.1. Optimum time for harvesting olives for crushing.

The quality of the olives, which is decisive in determining the oil quality, is strongly influenced by the variety and cultivation techniques applied to the olive grove. These, together, determine the oil content of olives and levels of the various components of its oil. The harvest time is directly related to the degree of ripeness of the olives.The mature olive passes through three stages of pigmentation following: green, semi-black and black. The degree of ripeness of the olives at the time of grinding affects both the quality and the quantity of the extracted oil.

Fruit color changes from lime green to pale green as fruit matures and changes color to purple and then black as it becomes fully ripe. The time of ripening will vary between varieties, environments, seasons and management practices.

• At the early stage of maturity (green stage), the olives are not rich in oil and give a finished product very susceptible to oxidation, due to its exceptionally high chlorophyll pigment, favoring oxidation in the presence of light. The oil from green olives is also rich in phenolic compounds (hydroxytyrosol and caffeic acid) endowed antioxidants properties. Add on that, fruity Green Oil is more easily oxidized due to the presence of omega 6 and omega 3 with 2 and 3 doubles bonds; this oxidation leads to a decrease in the phenolic fraction and thus, a change of tastes and flavors. The concentrations of naturally occurring phenols in fruity green oil are high that's why it will rancid slowly but the oil will remain consumable with a free acidity less than 0.8g/l (Granier, 2005).

• A full maturity stage (black stage) has a negative influence on the rates of polyphenols and aromatic compounds which are responsible of the sensory attributes of the oil and its stability to oxidation. It also promotes the fell of the olives, whether in natural or induced conditions (rain, wind, attacks by olives pests). The resulting olive oil, less rich in phenolic compounds (antioxidant activity), tends to be more acidic. In fruity black oil, no variations in tastes and flavors are observed since they are very few at the base. The optimal duration of harvest should be shorter because the lack of phenols increases the risk of oxidation. In poor storage conditions, the rancid flavor can appear much earlier than in fruity green oil (Granier, 2005).

2.4.1.1. Maturity Index.

The oil quality depends on the maturation stage when olives were harvested. The degree of maturity affects the volatile compounds that give oil its unique sensory characteristics, its content of minor compounds, acidic composition, mainly palmitic, oleic and linoleic. The optimal harvesting time must be determined for each variety of olive taking into consideration the maximum concentration and the best quality of oil in the fruit. The index for the optimum stage of maturity of "Picholine marocaine" is around 4.0 to 5.0.

Thus, the early harvested fruit yield a green oil of high quality and very fruity with low acidity. While the oil obtained from olives harvested at a later date has a slightly higher acidity, has a straw-yellow color, is soft and fruity but sometimes it has a dry taste, or even mold.

The quality of virgin olive oil is related to its chemical composition. Good oil should be a balance of its predefined acidity, levels of vitamins and relationships between minor components that determine organoleptic properties. The chemical composition of oil varies not only according to the olive variety, soil and climatic conditions but also with many factors relating to the cycle of production, processing, storage and packaging of the oil. The time and manner of collection, the storage time at the grove, the transportation conditions of the fruit, the storage duration before processing, the extraction process and the storage conditions will affect the composition of the final product (CI, 2001).

Qualitative characteristics of olive oil derived from the concomitant action of agronomic and technological workers in various operations from the collection of olives on the tree until the oil extraction (Khlif and Rekik, 1996; Di Giovacchino, 1996). Hitting the branches may cause injuries on olives and the fall of the fruiting twigs that must bear the fruit the following year. The acidity of the oil increases causing an alteration to the oil, the taste profile and the flavor while the productivity of the olive tree is compromised (CI, 2001).

Moreover, researches (Cimato, 1990; Khlif *et al.*, 1996; Garcia *et al*. 1996;-Kammoun Grati *et al*., 1997.; Ryan *et al*., 1998) have shown that oil quality depends fundamentally on the cultivar-environment interaction, especially the maturity of the olives. Physiological changes, directly related to the age of the fruit maturity, occur when changing the oil quality (Marzouk and Cherif, 1981; Ben Salah *et al*., 1986).

2.4.1.2. Moisture content.

Moisture content of olives changes dramatically from day to day. If the moisture content in the olive is high, the fruit will be heavy and the oil concentration will be diluted. There may also be a loss of flavor and reduced levels of antioxidants including polyphenols. Although the oil content does not change, the percentage of oil will vary from day to day based on the moisture content. It is therefore important to test the moisture content of the olives at the same time that oil content is tested so that samples can be accurately compared with each other and over time.

The fruit's water content influences the percentage of oil relative to moisture, so dried fruit will have a higher percentage of oil by weight. The extractability of the oil from the fruit is heavily

influenced by fruit moisture content, maturity, and many specifics of the extraction process such as paste fineness, malaxation time and temperature, and extraction machinery type (Vossen, 2007).

Fruit moisture content is often 55% or higher in developing fruit but then drops to 50% or less with the commencement of ripening. This will vary between varieties and seasonal conditions and some varieties will not follow this trend. Manzanillo will tend to maintain a high moisture level throughout the ripening period. By monitoring fruit moisture levels from the beginning of ripening period, growers can use moisture content to indicate optimum harvest timing and determine if it is conducive to good extraction and separation efficiency.

This is a measure of the water that may be present by placing a measured weight of oil in a drying stove for 120 minutes each time and measuring the weight difference. It is expressed as a percent (%) of the total weight (IOC, 2003b).

It is difficult to extract the oil from fruit that has been over-irrigated and has high moisture content. Fruit with low moisture has a higher percentage of oil and that oil is easier to extract (Vossen, 2007).

2.4.1.3. Influence of storage on the stability.

Depending on storage conditions, olive oil is subject to oxidation which can have a greater or lesser impact on taste and nutritional properties. It was shown that the way to store olive oil had an influence on his composition of phenolic compounds and fatty acids. Morello *et al.* measured changes, during 12 months, in olive oil stored under defined conditions of temperature and light (amber bottles, stored in the dark at room temperature) (Morello *et al.*, 2004). Results showed that after 12 months storage, there was first an increase (3%) of oleic acid, due to a decrease in polyunsaturated fatty acids (30% of omega 3 in less and less 10% omega 6).

The grinding and mixing (which can last from 20 to 40 min) represents a point key in the oil quality. First, during mixing, the water temperature must be below 27°C; that olive oil can be

described as "cold pressed". Indeed, high water temperatures cause changes in taste olive oil (Perez *et al*., 2003). In addition, the mixing time also plays an important role in quality taste and nutrition of olive oil.

The improper storage of the olives is the main cause of deterioration in the quality of the extracted oil. These alterations are due to the unfavorable enzymatic activity of the lipolysis, and the microbial growth during storage; with increasing storage time, an increase in the peroxide index and a deterioration of the organoleptic properties of the oil will take place, that's why olive should be stored in ventilated silos, filled in plastic boxes, with the use of fungicides, in a cold controlled atmosphere. The only way to limit the alteration of olives is to reduce the duration of storage (maximum 2-5 days) and to ensure best storage conditions of the olives by limiting the thickness of the olives layer to 20-30 cm thick for good aeration. The storage in the bags should be prohibited.

Oxidative rancidity is the main cause of deterioration of olive oil during storage. This is due to the reaction between the unsaturated fatty acids (regardless of whether they are in their free or esterified form of triglycerides), and oxygen, U.V, etc… This oxidation can be limited by keeping the oil away from heat and light (Kristott, 2000). The two factors that determine the susceptibility of the oil against the oxidation are fatty acid composition and antioxidant compounds. The type of fatty acids in the oil (the number of double bonds) determines the type and number of reactions that will occur during storage. The oleic acid (C18:1), present up to 83% in some olive varieties, has only one double bond, thus limiting the oxidation risks.

The extra virgin olive oil contains varying amounts of antioxidants. This includes carotenoids, tocopherols and phenolic compounds, with various amounts, giving the oil a defense against the free radicals. Studies have evaluated the contribution of these compounds to fight against the oxidation of the oil. It is estimated that stability of the oil depends on 30% of phenolic compounds, 27% fatty acids, 11% of vitamin E and 6% of carotenoids (Aparicio *et al.*, 1999).

The extraction of oil should begin within 1-3 days after fruit harvest to avoid deterioration of the flavor and any increase in the content of free fatty acids.

The amount of oil extracted from a given amount of fruit depends on many factors (Vossen, 2007):

•Oil content of the fruit varies by year, amount of fruit on the tree and variety.

•Extractability of the oil from the fruit varies by year, water content, fruit maturity and variety.

•Extraction process varies by paste fineness, malaxation time and temperature; decanter efficiency and/or the amount of time and pressure used on the press cake.

The olives are washed, crushed and kneaded into a smooth paste from which oil is extracted with cold, mechanical pressing or centrifugation. The vegetable water, a mixture of vegetation water and oil, are left to rest and the oil is extracted by decantation, centrifugation and filtration. The oil obtained exclusively by these mechanical processes, i.e. without heating, is called virgin olive oil (Van der Vossen *et al*., 2007).

2.4.2. Classification of olive oil.

The quantity of oil in the fruit is a built-in genetic factor, but it can vary from year to year due to tree vigor, crop load, fruit maturity, and fruit moisture content. Oil content varies by variety from less than 10% to about 30% on a dry weight basis. Since oil accumulation peaks when the fruit is quite mature, delaying harvest until the fruit is ripe assures the highest yield of oil, though it will change some flavor characteristics, and extractability if the weather is rainy (Vossen, 2007).

Olive oil is produced by mechanical means with the exclusion of solvents or reesterification process; as there are many varieties of olives, there are also several types of oils which now have clear and transparent standards, established by the European Community and covered by Council Regulation (EEC). In the EU, the virgin olive oil is classified into 4 categories on the basis of various characteristics, content of free fatty acids and the score on an organoleptic test, and classified as follows:

2.4.2.1. Virgin Olive Oils.

These oils are obtained solely from the fruit by mechanical or physical means under thermal conditions that do not lead to alterations in the oil (Vossen, 2007) using only treatments such as

washing, decantation, centrifugation, and filtration (IOC, 2007). The virgin olive oils are classified according to the free acid content, (g oleic acid/100 g olive oil):

i. ***Extra Virgin Olive Oil -*** Free fatty acidity (expressed as oleic acid) of an extra virgin olive oil should not exceed 0.8 grams per 100 grams (IOC, 2007). This is the highest quality rating for an olive oil. Extra virgin olive oil should have clear flavor characteristics that reflect the fruit from which it was made. In relation to the complex matrix of variety, fruit maturity, growing region, and extraction technique, extra virgin olive oils can be very different from one another (Vossen, 2007). This is an unrefined oil whose rating Sensory is equal to or greater than 6.5 and the others chemical characteristics are in accordance with EEC regulations.

ii. ***Virgin Olive Oil*** – Virgin olive oil which has a free fatty acidity (expressed as oleic acid), of not more than 2 grams per 100 grams (IOC, 2007). These are oils with analytical and sensory indexes that reflect slightly lower quality than extra virgin olive oil (Vossen, 2007). This is also unrefined oil whose rating Sensory is greater than or equal to 5.5 and the other chemical characteristics are in accordance with regulations of the European Community.

iii. ***Ordinary Virgin Olive Oil*** – Virgin olive oil which has a free acidity (expressed as oleic acid), should not exceed 3.3 grams per 100 grams (IOC, 2007), and the tasting notes is greater than or equal to 3.5. This is inferior oil with notable defects that is not permitted to be bottled under EU laws, so it is sent for refining. It will simply be absorbed into the lampante category. These oils come from bad fruit or from improper handling and processing. It is designated as not fit for human consumption (Vossen, 2007).

2.4.2.2. Refined Olive Oil.

Virgin olive oil that does not fit for human consumption as it is, designated lampante virgin olive oil: virgin olive oil having a free acidity (expressed as oleic acid), more than 3.3 grams per 100 grams and tasting notes are below 3.5. It is intended for refining or for technological use (IOC, 2007). This is oil obtained from virgin oils by refining methods that do not alter the initial glyceride structure. It has a free acidity of less than 0.3 and must be conformed to the other standards within its category.

Refined olive oil must not come from a solvent extraction of pomace. The refining process usually consists of treating virgin oil/lampante with sodium hydroxide to neutralize the free acidity, washing, drying, odor removal, color removal, and filtration. In the process, the oil can be heated to a high temperature as 220ºC under a vacuum to remove all of the volatile components (Vossen, 2007). Refined olive oil is usually odorless, tasteless, and colorless. It does not fit for human consumption.

2.4.2.3. Olive Oil.

These are oils that are a blend of refined and unrefined virgin oils. It must have a free acidity of no more than 1%. This grade of oil actually represents the bulk of the oil sold to the consumer on the world market. Other blends with more color and flavor would contain more virgin or extra virgin olive oil.

The virgin oil lamp and the oil obtained by heating or solvent extraction are intended for industrial use or to be refined by neutralization, bleaching and deodorizing to get refined olive oil. Meal or pomace obtained after cold extraction can undergo solvent extraction which provides pomace oil, type destined for industrial use (Van der Vossen *et al*., 2007).

2.4.3. Olive oil indices.

The determination of edibility of the olive oil is studied by the determination of its acid number, peroxide, saponification, iodine.

i. Acid Index (FFA) is the weight (mg) of potassium hydroxide required to neutralize 1 gram of fat. The presence of free acids in oil undermines conservation and stability during their heating.
ii. Peroxide Index indicates the amount of active oxygen capable of releasing iodine from iodides. The peroxide is the total amount of peroxides expressed as milliequivalents of oxygen per kg fat in the sample that oxidize potassium iodide under specific conditions of analysis. The oxygen in the air forms the CH_2 group adjacent to a double bond group OOH hydroperoxide. Polyunsaturated fatty acids are more sensitive to oxygen as monounsaturated fatty acids.

iii. Saponification Index is the weight of KOH needed to transform fatty acids into soap (necessary to saponify 1g of fat). It indicates the amount of free acids in 1 g of fat.
iv. Iodine Index is the number of grams of iodine bound per 100 g of fat. Iodine index increases with the proportion of unsaturated fatty acids

Moisture in oil promotes the breakdown of fatty acids, which lower its quality.

2.4.3.1. Free fatty acids.

Free fatty acid is the simplest and indicative test for good harvesting and handling processes prior to milling. The fatty acid chains, that had a hydrolytic breakdown, liberate glycerides (diglycerides and monoglycerides). Their presence indicates that degradation has occurred in the oil through poor handling during processing. Free fatty acids can influence the organoleptic value of the oil. Additionally, free fatty acids are water-soluble and may be lost during processing.

The IOC standard for free fatty acids in extra virgin olive oil is a maximum of 0.8%. If the FFA is high, e.g. over 0.8%, it indicates that there has been fruit damage (frost, bruising), delays between harvest and processing or harvesting of over-ripe fruit. If FFA is high, there are likely to be other problems with the oil and extraction may not be advisable. This may be the case if the fruit is frost damaged, as the oil will have a characteristic flavor due to a high content of FFA.

It is determined easily with a titration of potassium hydroxide that neutralizes the acidity. It is usually expressed as percent (%) free fatty acids on the basis of the oleic acid, because that is the predominant fatty acid in olive oil. It is commonly called the percent acidity or free acidity percent (IOC, 2003b).

2.4.3.2. Iodine index.

The iodine index of a fat is a measure of its degree of unsaturation. In fats containing saturated fatty acid, the iodine number is 0 because it contains no double bonds and no reaction takes place while this index is higher in unsaturated fat that contains double bonds; this is the case of olive oil containing unsaturated fatty acids (oleic, linoleic and linolenic).

The iodine number for oils is given in ranges because the fatty acid content depends on the growing conditions and variety. The higher the iodine number is, the more unsaturated the fat. The iodine number of a fat ranges from 0 to 70; while the iodine number of oil is greater than 70. The IOC standard of iodine index varies between 75 and 94 for virgin olive oil (Codex Alimentarius, 2003)

2.4.3.3. Peroxide index.

Oxidation of unsaturated fatty acids results in the formation of hydroperoxides. This phase is called initiation of lipid peroxidation. These hydroperoxides are unstable and can react with other molecules to generate new radicals. This is the propagation step in the acid peroxidation fat (Veillet, 2010).

Topallar *et al.* in 1997 showed that the oxidation of fatty acids depends directly on the ability of oxygen to react with peroxide radicals. The dosage allows having a report on the progress of the oil oxidation.

Peroxide value is the related test to the oil storage. This is a crude indicator of the amount of primary oxidation that has occurred, forming peroxide compounds within the oil. It measures chemical products that are produced through reaction with oxygen to ultimately cause rancidity.

Once the process of oxidation that leads to rancidity starts, it happens very quickly. The IOC standard for PV is $\leq$ 20 mEq O_2 /kg oil. A high value indicates that the olives or paste was handled improperly, the oil could be defective, and the oil might not keep well. In general, peroxide levels higher than 10 may mean less stable oil with a shorter shelf life.

If low peroxide oil is extracted with high peroxide oil, the final product became rancid very quickly. Several factors increase the peroxide value and include high temperature, light and oxygen. Contact with metal surfaces, such as copper, can also catalyze oxidation of the oil. The fruit damage and delays between harvest and processing are also a risk.

Oil should be stored in cool, inert vessels, such as stainless steel or glass, away from the light.

Oil, with a low peroxide value, means that olive harvesting and oil processing were handled adequately to ensure that oil meets the IOC and EEC standards. It also means that oils will be more stable and are stored correctly; this will lead to an extended shelf life.

It is done through a titration that liberates iodine from potassium iodide and is expressed as a value in milliequivalents of free oxygen per kilo of oil (meq O_2/kg) (IOC, 2003b).

2.4.3.4. Saponification index.

The saponification index is the measure of the amount of the alkali required to saponify a definite weight of fat. The saponification is an indication of the average molecular weight (or chain length) of fat. For pure fatty acids, the saponification value equals the acid value. The higher the molecular weight of a fat, the higher is its saponification number.

The IOC standard for the saponification index ranges from 184 to 196 (Codex Alimentarius, 2003).

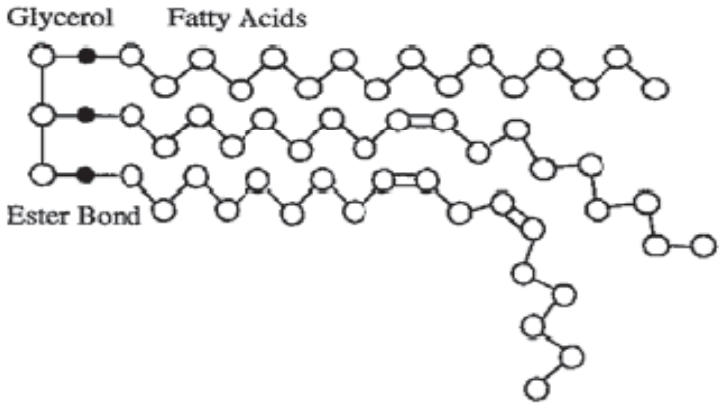
2.4.4. Fatty acid profile.

Fatty acids belong to the family of lipids. These lipids contain a saponifiable fraction called (phospholipids, triglycerides) and a minor unsaponifiables fraction (sterols, fat-soluble vitamins, carotenoids). Lipids are characterized by their insolubility in water and solubility in organic solvents (Veillet, 2010). Fatty acids are organic molecules containing a carbon chain terminated by a carboxyl group.

The glycerol can have any three of several fatty acids attached to form Triglycerides. The carbon chains may have different lengths. They may be saturated, monounsaturated or polyunsaturated. It is the relative proportion of these that make oil different from another.

In nature, fatty acids are usually in the form of fatty acid triesters:

Glycerol+3fatty acids ⟶ Triacylglycerol + 3 H2O



In the case of the olive oil, triacylglycerides represent between 98% and 99% of the total mass. Few free fatty acids can be found and show an oxidation of the triester. The fatty acid composition is highly variable and depends on the variety, the production region and the influence of environmental conditions. Standards are fixed by placing upper and lower limits in the Codex Alimentarius on the proportions of each fatty acid.

The proportions of the different fatty acids can influence the stability of the oil as well as determining the nutritional value of the oil. Some fatty acids are considered better than others.

The fatty acid profile is the measure of the proportions of individual fatty acids in the oil and therefore an important part of the oil quality. Each oil has different qualities for different proportions of fatty acids. Olive oil has a low content of saturated fat and high unsaturated fatty acids. Unsaturated fatty acids are divided into two categories: monounsaturated and polyunsaturated fatty acids. In case of saturated fatty acids, its carbon chain is free of any carbon-carbon double bond.

It can also contain a double bond (MUFA) or more double bonds (PUFA). For unsaturated fatty acids, they are often referenced by the position of the first double bond from the terminal methyl group. There are two major PUFA found in olive oil: linoleic acid (n-6) (omega 6) and acid alpha-linolenic (n-3) (omega 3). These fatty acids are "essential" because they cannot be synthesized by humans and must be provided by food (Veillet, 2010).

The variability of fatty acids is relatively high, but on average, virgin olive oil consists to 72% monounsaturated fatty acids (MUFA), 14% of polyunsaturated fatty acids (PUFA) and 14% saturated fatty acids (SFA) (Harwood and Aparicio, 2000). The polyunsaturated fatty acids represent a significant fraction of the oil and are mainly composed of linoleic acid. Unsaturated fatty acids are generally cis; some fatty acids, which are products of industrial processes, may have a trans spatial configuration. The isomerization cis-trans will be much more easily with unsaturated fatty acid and a pushed heat treatment (Judd, 1994). Cardiovascular risks are associated with absorption of these acids in large amounts, however, natural trans fatty acids seem harmless to human health (Motard-Bélanger, 2003).

Oleic acid, the main monounsaturated fatty acid with one double bond, acquired its name from olive oil and accounts nearly 70% of fatty acids. Oleic acid (omega 9), which is the most desirable nutritionally, reduces the level of "bad" cholesterol in the blood (LDL cholesterol) and maintains the level of "good" cholesterol (HDL). It should not be confused with the acidity of oil, which is expressed in grams of free oleic acid per 100 grams of oil.

Linoleic acid or Omega 6, which is not synthesized by the body, is a polyunsaturated fatty acid with 2 double bonds. These molecules are very sensitive to oxidation which may cause premature rancidity of the oils (Veillet, 2010). Linoleic acid alters the biological components by integrating into lipoproteins and by incorporation into cell membranes that are protected from the free radicals. Linolenic acid (Omega 3), which is also not produced by the body, is a polyunsaturated fatty acid with 3 double bonds. Linolenic acid, with three double bonds, is the most chemically reactive and therefore undesirable from the view of stability. Omega 3 and Omega 6 need to be assimilated.

The ratio of Omega 3/ Omega 6, which is essential, should be less than or equal to 5.

Stability of the oil is influenced by the degree of saturation with polyunsaturated fats such as linoleic and linolenic acid being many times more prone to oxidation than the mono-unsaturated fat, oleic acid. There is an inverse proportional relationship between oleic acid and linoleic acid. Oil with high oleic acid of 80%, will be low in linoleic acid (4–8%). If oleic acid is low, around 65% then linoleic will be high (10–18%). Olive oil with high oleic acid is nutritionally preferable

and potentially more stable than low oleic olive oil. The IOC standard for oleic is 55–83%. The reason for this is that climate, season and variety influence the fatty acid profile. In general, cooler, high altitude environments will produce higher oleic acid levels than hot, low altitude environments.

Olive oil contains also a small amount of saturated fatty acids, stearic and palmitic (8-20%), known to increase levels of bad cholesterol. Palmitic acid is a saturated fatty acid and is also undesirable. The fatty acid profile is influenced by cultivar and environment.

The limits of variability of the content of major fatty acids of olive oil as percentage of total fatty acids as set by the International Olive Council (IOC, 2003b) are as follows. The mono-unsaturated oleic acid dominates (55-83%), followed by a small percentage of saturated fatty acids palmitic (7.5-20%) and stearic acids (0.5-5%) and an average percentage of polyunsaturated fatty acids, linoleic acid (3.5-21%) and a-linolenic acid (<1%).

The predominance of monounsaturated oleic acid, the low percentage of saturated fatty acids and an acceptable percentage of polyunsaturated fatty acids give the oil its great biological importance. The unsaturated fatty acids are sensitive to oxygen, resulting in a phenomenon of self-oxidation (Harwood and Aparicio, 2000). The rate of autoxidation is similar to the number of double bonds of fatty acids, whereas it is hindered by the type and quantity of antioxidants present in olive oil.

The remaining 1% is the minor compounds such as squalene (0.15 mg/100 ml, a substance that inhibits the synthesis of cholesterol), triterpene alcohols (increases the secretion of bile acids contributing to the elimination of cholesterol), sterols (β-sitosterol, a substance that maintain intestinal absorption of cholesterol), phenols (oxidation resistance), derivatives of tocopherol and chlorophyll (stimulates cell growth and accelerates the healing process). Some of these ingredients act as antioxidants that protect the body against damage that comes from oxidation by free radicals, while at the same time they protect the olive oil against oxidation (rancidity).

2.4.5. Phenolic compounds.

If fatty acids represent the vast majority of the composition of the oil in terms of mass, the minor compounds such as phenolic compounds play a very important role in the characterization of oils and their nutritional value (Brenes *et al.*, 2002; Visioli, 1998). Olive oil contains phenolic compounds and simple complexes that increase stability and confers antioxidant properties and modulate taste (Fedeli, 1977). Phenolic compounds contribute significantly to the spicy taste, the astringency and bitterness of oils (Brenes *et al*., 2000).

Phenolic compounds are highly variable from oil to another, both in quantitative and qualitative terms. It is generally accepted that the phenolic activity is between 200 and 600 mg of gallic acid equivalents per liter of oil.

The phenolic composition can serve as a marker for the identification of oils because the origin geography has a strong influence on the development of certain phenols (Vinha *et al.*, 2005). The second factor influencing the phenolic composition is the cultivation and maintenance systems including irrigation of olive trees (Gomez *et al*., 2009). Indeed, one water stress will lead to greater amount of phenolic compounds. Finally the most important factor of all is the variety of olives grown. Many Studies have shown that certain varieties of olives such as Cornicabra were richer in compounds phenolics than others such as Picolimon (Gomez, 2008; Tura, 2008; Vinha *et al.*, 2005).

2.4.6. Tocopherols.

The total content of tocopherols in olive oil is highly variable since it was reported in a range from few to 450 mg/kg of oil (Boskou, 2006; Gutierrez, 1999). Alpha-tocopherol alone accounts for 90% of total tocopherols (Sherwin, 1976), but there are also some beta and gamma tocopherol, delta tocopherol while this is only trace amounts (Psomiadou, 2000).

2.4.7. Olive oil and health.

The virgin olive oil plays an important role in the food industry and is important in human nutrition for several reasons. Olive oil acts favorably on the functioning of the liver, intestine and cardiovascular system. In countries that consume olive oil, the incidence of myocardial infarction

is lower than in the consuming countries of saturated fats (butter). More interest in olive oils has been increased since the discovery of their high fat-soluble vitamins value.

The mono-unsaturated, oleic acid, gives a measure of stability because it is not very sensitive to oxidation. Oleic acid exerts a favorable effect on growth and increases the absorption of calcium and vitamin D. They are also an important source of essential polyunsaturated fatty acids not synthesized by the human body. They are also present for their benefits, particularly in terms of protection against cardiovascular disorder and cancer. Its minor components, polyphenols, enable the authentication of oil (Veillet, 2010).

Research has shown that consumption of olive oil resulted in a reduction of bad cholesterol (LDL) without reducing the "good" cholesterol (High Density Lipoprotein); this leads to reduce the LDL / HDL ratio. Diet is one of several factors that may cause hypertension. Positive relationships were found between blood pressure and salt intake (Sack *et al*., 2001), excess saturated fatty acids (Stamler *et al*., 1996) and alcohol (Puddey *et al*., 1997). Regarding the qualitative aspect of fatty acids, it is known that supplementation of diet rich in polyunsaturated fatty acids (omega 3) resulted in a decrease in systolic and diastolic pressure in normal subjects as in subjects that have hypertension (Appel *et al*., 1993; Iacono *et al*., 1983). Thus, the MUFA-enriched diet would reduce blood pressure of 5 to 9% in healthy subjects and hypertensive subjects.

3. Material and Methods.

3.1. Plant Material collection.

Healthy shoots with a length of 12-15cm were collected in the morning, for two consecutive years (2009 and 2010) from branches of the current year growth from the millennium trees during four seasons of the year (spring, summer, autumn). These olive trees are used for the initiation of cultures from axillary buds.

The shoots were defoliated and then disinfected first by a regimen consisting of the benomyl followed by disinfection with bleach (NaOCl) at a concentration of 35%. One week after, all introduced explants (100%) were infected by a fungal mycelium and a high percentage of tissues browning caused by the high concentration of NaOCl appeared.

In order to reduce the rate of fungal and bacterial infections, the excised nodal segments of the second collect, were exposed to a mix of fungicides (propamocarb-HCl, Carbendazim, fosethyl-aluminium and methalaxyl+mancozeb) and bactericides, the NaOCl (15%) and Chloroxylenol (2.5%) in order to minimize the percentage of the browning tissues during the initiation phase. An antibiotic, the oxytetracycline (1mg/l), was added to the media to minimize the bacterial infection.

3.2. *In vitro* culture.

3.2.1. Plant material preparation.

Before the removal of leaves, and only one hour after their collect, the branches were soaked for three hour in a solution containing the propamocarb-HCl (2g/l) and methalaxyl+mancozeb (6g/l).

The plant material was transferred to the laboratory where the leaves were removed and then, twigs were divided into uninodal portions of 3 cm and disinfected according to the following protocol:

- Soaking in an agitated solution of carbendazim and fosethyl-aluminium (5g /l) for half an hour.
- Rinsing with water for 5 min.
- Rinsing in ethanol (70%) for 10 sec.

- Incubation in a solution of Chlorox (NaOCl 110g /l) diluted to 15% for 5, 10 and 15min.
- Rinsing three times successively in sterile distilled water under laminar flow hood for 5 min.

3.2.2. Fungicide treatments.

Fungicides that have been tested have two modes of action and are described below:
- Carbendazim and Mancozeb act preemptively against bacteria of the Pseudomonas family (pathogen causing bacterial blight of the olive tree) and other fungal diseases attacking the olive tree. This fungicide has systemic properties.
- Metalaxyl, Propamocarb-HCl and Fosethyl-Aluminium act cursively and preventively by inhibiting the spores and blocking the mycelium of several families mainly Cyclocconiums (pathogen causing scab of the olive tree, hitting almost all Lebanese olives). These fungicides have also systemic properties.

The above mentioned fungicides, with their mode of action that last up to three weeks, may prevent the development of mycelia after their introduction *in vitro*.

3.2.3. Preparation of culture media.

Three media were tested in this study (Tab.3) in all phases, initiation, multiplication, root and callus formation. The media tested were MS (Murashige and Skoog, 1962), Olive Medium (Rugini, 1984) and Woody Plant Medium (WPM) (McCown and Lloyd, 1981) (Table 2). They have the same macro and micro components, but differ in their concentration as well as their hormonal balance. The choice of hormonal balance in terms of absence or presence of auxin and cytokinin was based on the stages of culture.

The media were solidified by Bacteriological agar (0.8%) after adjusting the pH to 5.8 with HCl or NaOH. The autoclave was held for 20 min at 118°C.

The media were left either in test tubes (15ml/tube) or in jars (25ml/jar). In case of antibiotic application, the medium previously sterilized by autoclaving was sunk under the laminar flow hood. The composition of media is shown in table 2.

Table 2. Basal nutrient medium composition of Murashige and Skoog medium (MS), olive medium (OM) and woody plant medium (WPM).

Componants	MS (mg/l)	WPM (mg/l)	OM (mg/l)
Macro-éléments			
$CaCl_2.2H_2O$	440	96	0
$Ca(NO_3).4H_2O$	0	556	1300
KH_2PO_4	170	170	340
KNO_3	1900	0	1772
$MgSO_4.7H_2O$	370	370	731
NH_4NO_3	1650	400	412.5
K_2SO_4	0	990	0
Micro-éléments			
$CaCl_2.6H_2O$	0.025	0	0.02
$CuSO_4.5H_2O$	0.025	0.25	0.25
H_3BO_4	6.2	6.2	12.4
KI	0.83	0	0.83
$MnSO_4.H_2O$	22.3	22.3	16.9
$NaMoO_4.2H_2O$	0.25	0.25	0.25
$ZnSO_4.7H_2O$	8.6	8.6	14.3
Fer			
$FeSO_4.7H_2O$	27.8	27.8	27.8
Na-EDTA.H_2O	37.3	37.3	37.5
Vitamines			
Inositol	100	100	100
Pyridoxine	1	0.5	0.5
Thiamine	1	1	0.5
Acide nicotinique	1	0.5	5
Acide ascorbique	25	25	0
Glycine	0	2	2
Biotine	0	0	0.05
Acide folique	0	0	0.5
Glutamine	0	1178	0
Others			
Saccharose	30000	30000	30000
Agar-Agar	8000	8000	8000
H_2O	QNF1L	QNF1L	QNF1L

3.2.4. Initiation phase.

Under the laminar flow hood, disinfected fragments of olive (1-1.5cm long), each bearing two axillary buds (nodes) were cultured in jars containing 20 ml of culture medium with two buds / jar. The media MS based on that of Murashige and Skoog (1962), the OM (Olive Medium) and WPM (Woody Plant Medium) were used. These medias were supplemented with different concentrations of BAP (2, 3 and 4mg/l), the growth regulator that enhances axillary proliferation of explants. After adjusting the pH to 5.8 and the addition of agar, the media were sterilized by autoclaving at a temperature of 118 ° C for 20 min.

For each media, 30 cuttings were introduced. Cultures were maintained in a growth chamber conditioned at a temperature between 20 and 22ºC and a photoperiod of 16h/day with a light intensity of 4000 lux provided by fluorescent lamps.

The ability of these explants to survive the initiation phase was evaluated by measuring the newly developed explants, shoot length. The survival rates, as well as percentage of different infections (bacterial and fungal) and tissues browning, were also evaluated.

3.2.5. Cultural maintenance and multiplication.

Five weeks after the establishment phase of buds, new shoots developed, each bearing one and two on average. The newly developed buds were divided into nodal explants after their separation from the initial explant. These microcuttings were transferred on the same media added with same concentrations of cytokinin (to promote the development of axillary buds) forming the first subculture. This operation was repeated five weeks after the first subculture and seedlings were transferred to new media to explore the influence of hormones on the shoot proliferation and multiplication rate. The multiplication rate, which is the ratio between the numbers of new shoots developed on the initial number of shoots, was evaluated at the end of each subculture.

New shoots were transferred and grown for four continuous subcultures on the same media. Multiplication phase was held under the same conditions ensured during the initiation phase.

3.2.6. *In vitro* Rooting.

Rooting was induced by auxin shock method after an elongation phase where explants were transferred on the same media without adding any hormone; the rooting media, Murashige and Skoog (1962) media, the olive media (OM) (1984) and WPM were supplemented with different auxins, NAA and IAA (2mg/l), in order to promote rooting. The rooting rate was studied five weeks after the transplantation of micro cuttings on new media.

The proliferation and root development was made in the light under 16 hours of photoperiod and a temperature was maintained at 25 ± 2°C. The explants were then transferred to a fresh medium devoid of auxins to promote root development.

3.2.7. Callogenesis.

During autumn and four weeks after the initiation phase, calluses appeared at the base of explants. These explants were wounded at the base and transferred on a development media (MS medium, added with NAA (2mg/l) and BAP (0.5mg/l)), to encourage the development of somatic embryos and subsequent regeneration into roots and plantlets. The explants were kept in the dark at 25ºC for one week before being subject to the conditions of the growth chamber with a photoperiod of 16h/d and a light intensity of 4000 lux.

The percentage of explants that developed callus was compared between three seasons (spring, summer and autumn) in order to study the influence of the season on the callus formation.

3.8. Culture conditions.

During the different stages (initiation, multiplication, rooting), cultures were maintained in a controlled room with specified temperature conditions (20-22°C), photoperiod (16 h/d) and light intensity (3000-5000 lux). In the case of callus, the material was kept in the dark for one week before putting it back under the same conditions of the culture chamber.

3.2.9. Acclimatization.

This phase consisted on the evaluation of the micropropagated plants and their potential adaptation to the environment. The rooted plantlets were removed from jars and the roots were washed to remove the agar; then, they were transplanted into plastic pot (8cm) containing an irrigated mix of potting soil and peat moss (1:3).

Carbendazim, which is a fungicide, was added to prevent any deterioration of roots. The newly transferred plants were put in a table covered with a plastic film (50µ) in order to conserve high relative humidity (around 90%). The plastic film was partially removed one week after the introduction of the rooted plants.

The non-subcultured explants that developed roots and a minimum of two leaves were transplanted into pots containing a substrate composed of peat and vermiculite (4:1). Acclimatization took place in the nursery under controlled environmental conditions.

3.3. *In vivo* culture.

Cuttings were introduced *in vivo* in parallel of the introduction of explants *in vitro*. First, the basal end of cuttings was dipped in a powder formulation with 0,8% of naphthalene acetic acid (NAA); then, cuttings were placed in alveolar containers filled with propagation medium composed of a potting soil and peat moss mix (1:3). These containers were placed in small tunnel structure (7m*1.4m) covered with a double layered shading in order to minimize direct sunlight and transpiration of cuttings. The relative humidity ranged between 80 and 90% and the temperature was maintained at 20-24ºC using heaters to increase temperature during winter and fans to decrease it during spring and summer.

The percentage of survival, shoot length, callus formation and rooted cuttings were evaluated, respectively 30, 45 and 60 days after their introduction under the tunnels during the four seasons and for two consecutive years (2009 and 2010). The numbers of newly developed leaves were also determined. In order to induce hardiness of the rooted plants, cuttings were exposed to lower humidity by opening gradually the two layers until reaching the same relative humidity which is around 40-45%.

3.4. Olive parameters

Olives were collected on weekly basis during the maturation phase starting the first week of October till the second week of December during the seasons of 2009 and 2010. The selected drupes, healthy and free from pests and diseases, were conserved in perforated plastic bags in a refrigerator at 4ºC until tested.

3.4.1. Fruit weight and size (length and width).

3.4.1.1. Fruit weight.

Thirty olives from each sample were randomly selected and weighed using an analytical scale with gradation of 0.01g.

3.4.1.2. Fruit length and width.

The length and width of thirty olives randomly selected olives from each collect was measured using Vernier calliper.

3.4.1.3. Pit weight.

The pit is separated from the flesh and weighed on an analytical scale with gradation of 0.01g.

The average weight, width, length of the fruit and pit weight at each harvest were subsequently calculated.

3.4.2. Shape index.

The shape index, of a random sample of thirty olive fruits, is calculated as follows (Buyanov and Voronyuk, 1985):

I = i/d

Where:

I: shape index.

i: length of fruit (mm).

d: diameter of fruit at the middle of its length (mm).

3.4.3. Flesh/ pit ratio.

The flesh content was calculated by subtracting the pit weight from the whole olive fruit weight. The flesh to pit weight ratio was determined by dividing the flesh mass by the pit mass. This ratio was calculated for individual fruit using the weight of fruits and its pit as follows (Mohsenin, 1984):

Flesh/pit ratio = (Wf – Wp) / Wp

Where:

Wf: weight of the individual olive fruit (g)

Wp: weight of pit for the same olive fruit (g)

Apparatus

1. Digital Vernier calipers with accuracy of 0.01mm was used for measuring length, diameter and flesh thickness.
2. Electrical balance with an accuracy of 0.001g.
3. Stainless-steel knife.

Procedure

The pits were weighed after the horizontally cut of the fruits with a stainless steel knife.

3.4.4. Maturity index.

The maturity index was determined on 50 randomly selected olives in each sample to obtain a numerical value for the olive sample appearance. The olives were sorted into categories using the following parameters:

0 = Skin is a deep or dark green color.
1 = Skin is a yellow or yellowish-green color.
2 = Skin is a yellowish color with reddish spots.
3 = Skin is a reddish or light violet color.
4 = Skin is black and the flesh is completely green.
5 = Skin is black and the flesh is a violet color halfway through.
6 = Skin is black and the flesh is a violet color almost through to the stone.
7 = Skin is black and the flesh is completely dark.

Maturity Index (MI) = (0 x no) + (1 x n1)…+ (7 x n7) /100

3.4.5. Moisture content.

For every collect, approximately 60-70g of fruit was crushed using a hammer mill after the stones removal. Then, the paste was transferred to a previously weighed Petri dish. The sample was then transferred to a fan-oven at 105ºC for 2 hours. The dried sample removed from the oven is placed in a dessicator and cooled to room temperature. The dry weight of the sample is then recorded. The moisture content of the fruit is calculated as a percentage of the fruit weight. The dry matter (DM) of a feed contains all the nutrients except water. It is indirectly determined from the moisture content of the feed. After determining the moisture content, dry matter is calculated to be the difference (AOAC, 1995).

Apparatus

1. Analytical scale with gradation of 0.01g.
2. Oven with air flow of 60°C - 105°C.
3. Desiccator.
4. Spatula or spoon.
5. Petri dishes.

6. Knife.

7. Food processor with chopping blade.

Procedure

The empty dish was dried in the oven at 105ºC for 3hours and then transferred to desiccator to cool and then weighed. Then about 3g of sample are weighed and spread with spatula in the dish. After that, the dish is placed with the sample in the oven where they are dried for 3 hours at 105ºC. After drying, the dish is transferred to the dessicator. Once cooled, the dish and its dried sample were reweighed.

Calculation

Moisture content (%) = (W1-W2)*100/W1

Where,

W1= weight (g) of sample before drying.

W2= weight (g) of sample after drying.

3.5. Olive oil parameters.

No more than 72h elapsed between harvesting and pressing to avoid the risk of fermentation and development of defects in the oil. All analyzes were performed according to IOOC standards.

3.5.1. Olive oil content.

The oil is extracted in the laboratory by a soxhlet machine (Soxhlet SER148, solvent extractor, serial number 66987) using n-hexane as solvent on the dried sample of olive paste.

Calculation

Oil content (%) = W2/W1*100

Where,

W1= fresh weight (g) of the sample.

W2= weight (g) of extracted oil.

3.5.2. Determination of Acid Value (IUPAC, 1979).

The acid value was calculated for each sample collected starting October till December.

Definition

The acid value is defined as the number of milligrams of potassium hydroxide required to neutralize the free fatty acids present in one gram of fat. The value is expressed as percent of free fatty acids calculated as oleic acid.

Apparatus

1. Analytical balance.
2. 250 ml conical flasks with stopper.
3. 50ml test tube.
4. Burette.

Reagents

1. Standard aqueous potassium hydroxide or sodium hydroxide solution 0.1 or 0.5N.The solution should be colorless and stored in a brown glass bottle.
2. Ethyl ether.
3. Ethanol (95% in volume).
4. Phenolphthalein indicator solution (1.0 g of phenolphthalein dissolved in 100 ml of Ethanol 95-96%).

Procedure

Five grams of oil are weighted accurately in a 250 ml conical flask and then 50 ml of freshly neutralized hot ethyl alcohol (25ml+25ml) and about 1 ml of phenolphthalein indicator solution are added. The mixture is then boiled for about 15 minutes and titrated with the potassium hydroxide solution until the indicator changes to light pink and the color remains for at least 10 seconds.

Calculation

Acid value = 56.1V*N/W

Where,

V = Volume in ml of standard potassium hydroxide or sodium hydroxide used.

N = Normality of the potassium hydroxide solution or sodium hydroxide solution.

W = Weight in g of the sample.

The acidity is frequently expressed as free fatty acid for which calculation shall be Free fatty acids as oleic acid = 28.2 VN per cent by weight W

Acid value = Percent fatty acid (as oleic) x 1.99

3.5.3. Determination of Fatty acid composition of Oils and Fats by Gas Liquid Chromatography (EC, 2002).

For the determination of fatty acid composition, Fatty Acid Methyl Esters are formed by trans-esterification of the fatty acids with methanolic potassium hydroxide and, separated by gas–liquid chromatography equipped with an MS detector. The pattern of methyl esters is then compared with authentic oils for identification. This mode is suitable for neutral oils and fats with an acid value less than 2.

This rapid method is applicable to olive oils and olive-pomace oils with a free fatty acid content of less than 3.3%. Free fatty acids are not esterified by potassium hydroxide.

Apparatus

1. Gas liquid chromatograph (GC.320070395) with the following specifications:

1.1. A split type injection system.

1.2. Oven.

The oven, capable of heating the column to at least 220°C, maintains the desired temperature to within 0.1°C.

1.3. Capillary column.

1.3.1. Column.

Thermo TR-5MS (length 30 m, internal diameter 0.25 mm, with a stationary phase coating thickness of 0.25µm) (Part number P/N: 260F142P, serial number S/N 1104923 G8).

1.3.2. Stationary phase of moderate polarity. Phenyl Polysilphenylene-Siloxane (5%).

1.3.3. MS Detector.

2. Test tubes (5ml volume) with cap.

3. Automatic pipette.

Reagents

For the sample preparation

1. Methanol containing not more than 0.5% water.

2. Hexane chromatographic quality.

3. Potassium hydroxide approximately 2N methanolic solution. Dissolve 11.2g potassium hydroxide in 100ml of methanol containing not more than 0.5% m/m water (anhydrous methanol).

4. Nitrogen, containing not more than 0.5mg/Kg of oxygen.

For the chromatograph

1. Carrier gas: helium.

2. Auxiliary gas: hydrogen 99.9 per cent minimum, free from organic impurities; air or oxygen, free from organic impurities.

3. Reference standards of lipids of known fatty acid composition corresponding to that of the sample. Otherwise, reference standards of individual fatty acids, or fatty acid methyl esters.

Procedure

In a 5ml screw-top test tube, approximately 0.1g of the oil sample is weighed; then, 2ml of hexane are added, and shacked. After that, 0.2ml of 2N methanolic potassium hydroxide solution is added, and the cap fitted with a PTFE joint is put, tighten, and shacked vigorously for 30 sec. Leave to stratify until the upper solution becomes clear. The upper layer containing the methyl esters is decanted. The hexane solution is suitable for injection into the gas chromatograph. It is advisable to keep the solution in the refrigerator until gas chromatographic analysis. Storage of the solution for more than 12 hours is not recommended.

Because the oil includes fatty acids containing more than 2 double bonds, it is advisable to purge the air from the methanol and the flask by passing a stream of nitrogen into the methanol for a few minutes.

Determination of fatty acids

GC is programmed to maintain column temperature on 185ºC and detector temperature at 200ºC; 0.1–2µl of 5-10 % of Hexane solution of methyl esters is injected.
Initial temperature: 150ºC.
Final temperature: 210ºC.
Flow through the column: 1ml/min.
Injection volume: 1µl.

A reference standard mixture of known composition is analyzed under the same operating conditions as those employed for the sample and the retention distances or retention times is measured for the common fatty esters.
The peaks from the sample are identified from the graph. Fatty acids appear on the chart in increasing number of carbon atoms and increasing unsaturation. Thus C16 appears before C18, C18:1 before C18:2 and so on.

Chromatographic peaks were identified by comparison with the retention times of methyl esters of the constituent fatty acids purchased from Labotech (USA).

In order to compare Bchealehs' oil to ISO certificated oil (Boulos Oil) and oil from the Soury cv. planted at same altitude; and to evaluate the influence of storage time on quality, different samples of oil were analyzed. The determination of edibility of the olive oil is studied by the determination of its acid number, iodine, peroxide, saponification after 3, 6, 9 and 12 months of storage.

3.5.4. Determination of Iodine Value (AOAC, 2000).

The iodine value is a measure of the amount of unsaturation (number of double bonds) in a fat. The iodine value of oil is the number of grams of iodine absorbed by 100g of the oil, when determined by using Wijs solution.

Apparatus

1. Analytical balance.
2. 500 ml Erlenmeyer flask.
3. 50 ml test tube.
4. 25 ml pipette.
5. Burette.

Reagents

1. Wijs Iodine monochloride solution.

1.1. Iodine mono-chloride dissolved in glacial acetic acid, free from ethanol (Hanus).

1.2. Chloroform (CHCl3).

2. Potassium iodide (free from potassium iodate) - 10% solution prepared fresh.
3. Standard solution of thiosulfated sodium (0.1N).
4. Starch solution (1g of Starch dispersed in 100ml of distilled water).

Procedure

An appropriate quantity of the oil (150-200mg) is weighed accurately, into a 500 ml conical flask to which 10 ml of chloroform have been added. The content is mixed well and added by 25 ml of Hanus solution. The flasks are kept in dark for half an hour for non-drying and semi-drying oils and one hour for drying oils. After standing, 25 ml of potassium iodide solution are added, followed by 150 ml of recently boiled and cooled water. Before titration, 1ml of starch solution was added as indicator. Finally, liberated iodine is titrated with standardized sodium thiosulphate solution (0.1N), at the end until the blue color formed disappears after thorough shaking with the stopper on.

Blank determination is conducted in the same manner as test sample but without oil/fat.

Calculation

Iodine value = 12.69 (V0-V) N/W

Where,

V0 = volume in ml of standard sodium thiosulphate solution required for the blank.

V = volume in ml of standard sodium thiosulphate solution required for the sample.

N = normality of the standard sodium thiosulphate solution.

W = weight in g of the sample.

3.5.5. Determination of Peroxide Value (Horwitz, 2002).

The peroxide value is the quantity of peroxides in the sample, expressed in terms of milliequivalents of active oxygen per kilogram (mEq O_2/kg), which oxidize potassium iodide under the operating conditions of the analysis. This helps in assessing the amount of oxidation produced in the oil after its exposure to air.

Apparatus

250 ml Erlenmeyer flask.

Reagents

1. Acetic acid.
2. Chloroform ($CHCl_3$).
3. Potassium Iodide.
4. Sodium Thiosulfate ($Na_2S_2O_3$, 0.1N).

Procedure

Sample of 5.00g (+/- 0.05 g) are weighed into a 250 ml Erlenmeyer flask and then 30 ml acetic acid - chloroform (3:2) solution (under the hood) were added. The flask is swirled until the sample is dissolved and 0.5 ml saturated potassium iodide (KI) solution is added to the solution. Allow the solution to stand with occasional swirling for one minute and then added with 30 ml distilled water.

The titration is done slowly with sodium Thiosulfate ($Na_2S_2O_3$, 0.1N) adding it with constant and vigorous shaking. Titration is continued until the color changes to light yellow; 0.5 ml of 1% soluble starch indicator is added in order to give a blue color. The solution must be shacked vigorously near the endpoint which is a faint blue color to liberate all of the iodine from the chloroform ($CHCl_3$) layer. The sodium Thiosulfate ($Na_2S_2O_3$) is added drop-wise until the blue color just disappears.

Calculation

Peroxide value = S x M x 1000/ W

Where,

S = volume in ml of $Na_2S_2O_3$.

N = normality of the standard sodium thiosulfate ($Na_2S_2O_3$) solution.

W= weight of sample in grams.

3.5.6. Determination of Saponification Value (AOAC, 2000).

The saponification value is the number of mg of potassium hydroxide required to saponify 1g of oil/fat.

Apparatus

1. 250 ml capacity conical flask with ground glass joints.
2. 1 m long air condenser, or reflux condenser (65 cm minimum in length) to fit the flask.
3. Hot water bath or electric hot plate fitted with thermostat.

Reagents

1. Alcoholic potassium hydroxide solution dissolved in Ethanol 95-96%.
2. Phenolphthalein indicator solution (1.0 g of phenolphthalein dissolved in 100 ml of Ethanol 95-96%).
3. Standard hydrochloric acid: approximately 0.5N.

Procedure

The first step is weighting 2.0 g of oil in a 250 ml Erlenmeyer flask, then pipette 25 ml of the alcoholic potassium hydroxide solution into the flask. The sample flask is connected to an air condensers that is kept on a water bath; the flasks are boiled gently until saponification is complete (between 30-60minutes); A clear solution must appears with the absence of any oily matter, this mist be achieved within one hour of boiling. After the flask and condenser have cooled, the inside of the condenser is washed with 10 ml of hot ethyl alcohol neutral to phenolphthalein. The excess potassium hydroxide is then titrated with 0.5N HCl, using about 1.0 ml phenolphthalein as indicator.

Calculation

Saponification Value = 56.1*N (V0-V)/W

Where,

V0 = Volume in ml of standard hydrochloric acid required for the blank.

V = Volume in ml of standard hydrochloric acid required for the sample.

N = Normality of the standard hydrochloric acid.

W = Weight in gm of the oil/fat taken for the test.

3.6. Statistical Analysis.

The *in vitro* parameters corresponding to the average number of survivors and broken buds, explant contamination (bacterial, fungal and browning tissues), new stems/explant, number of new leaves formed, stem length, multiplication rate and percentage of explants that developed callus are calculated.

The *in vivo* results corresponding to the survival rate, number of newly developed leaves and length of the cuttings was also recorded, as well as the callus and root formation.

The olive parameters, weight, width, shape index, flesh/pit ratio, moisture and oil content, fatty acid composition and acid index were calculated along the ripening process, while the oil indices were recorded for one year at three months interval.

An analysis of ANOVA variance and correlation of all the mentioned parameters were performed using SPSS version 14.0 to examine the effects of different factors; culture media, subculture, season for the *in vitro* culture; cultivar, season, year and number of days after initiation were the factors that were studied in the *in vivo* culture; date of collection and production year for the olive and oil parameters; and finally the effect of the conservation on the quality parameters of olive oil.

4. Results and discussion.

4.1. *In vivo* culture.

In order to maximize the rooting, cuttings were kept for two months under optimal conditions of temperature (18-30ºC) and humidity (80-90%).

Cuttings, with a diameter less than 0.5cm and introduced during spring 2009, lost their viability six weeks after their introduction under the shaded polyethylene tunnels. Cuttings of same diameter, introduced during summer 2009, lost also their viability between the third and the fourth week after their introduction *in vivo*.

Only cuttings with a diameter that exceeded 0.5cm succeeded to overcome the initiation phase. For this reason, the highest survival rate and percentage of proliferated bud were observed on cuttings with a diameter >0.5cm during the three seasons, spring, summer and autumn (Figure 4).

Figure 4. Newly introduced cutting under double layered table.

The survival rate and percentage of proliferated buds

It was indispensable to study the effect of season on cuttings survival even if there was no roots emergence and development. Concerning Bchealehs cuttings introduced during the last week of September 2009, the percentage of proliferated buds was 66.67%, while the highest rate exceeded 88% during spring 2010 (Graph 1). For the spring and summer season of 2009, the rate was respectively 56.6 and 40%, while during summer and autumn 2010, it was 60 and 76%. For

Soury cuttings, the percentage of proliferated buds varied between 60 and 76.67%. All the proliferated buds started to emerge within one month.

The power of emergence and difference in number of proliferated buds is mainly due to the application of foliar fertilizer (20/20/20) during the period between May and October 2009 that led to a better vegetative growth on the millennium trees during 2010 (Figure 5).

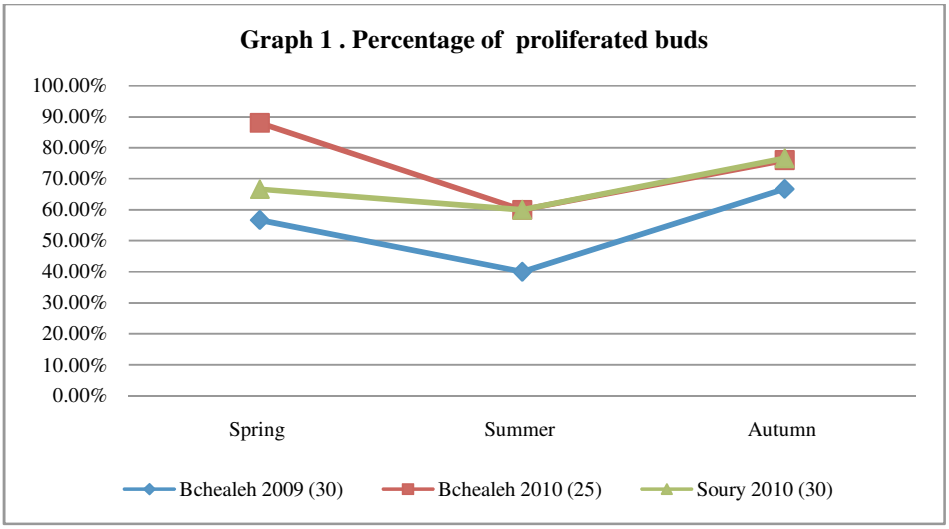
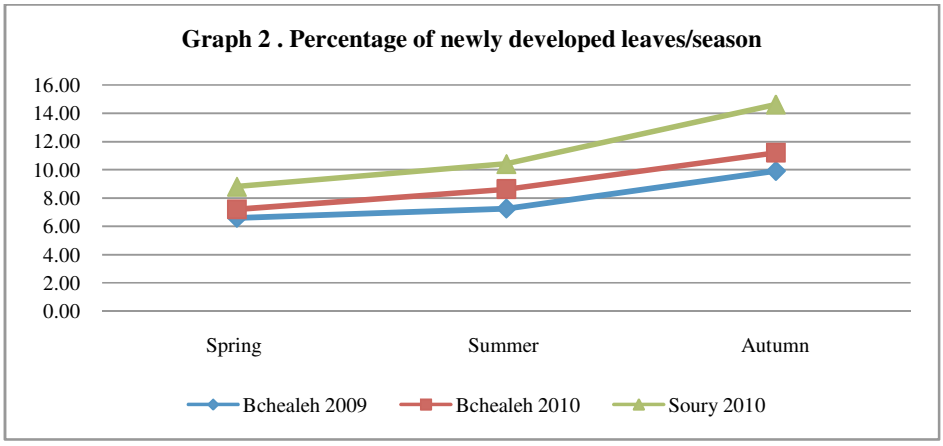


Figure 5. Buds emergence two weeks after the cuttings introduction

For the mean number of newly developed leaves in Bchealehs cuttings, autumn season gave the highest rates during 2009 and 2010 with respectively 9.9 and 11.2, followed by summer season. The lowest number of newly developed leaves appeared during spring with respectively 6.59 and 7.2 for the year 2009 and 2010. The Soury cuttings had the highest number of newly developed leaves with 14.6 during autumn 2011 (Graph 2).

Figure 6. Bchealehs cutting after five, six and seven weeks of its introduction *in vivo*



The newly developed leaves in the developed cuttings were highly significantly influenced by cultivar, year, season and days after initiation (Table 3).

Table 3. Effect of cultivar, year, season and number of days after initiation on the newly developed leaves.

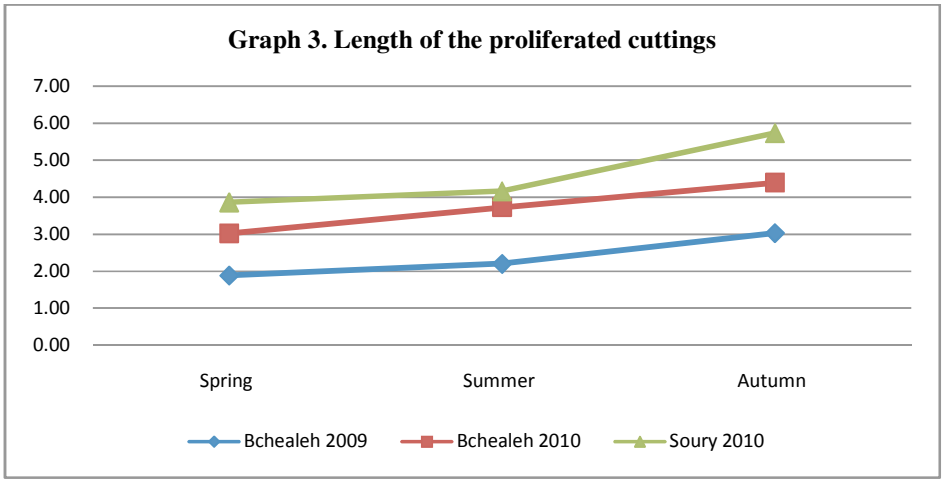
	Cultivar		Year		Season		Days after initiation	
	F	p	F	p	F	p	F	p
Number of newly developed leaves	25.287	0.000***	100.418	0.000***	52.823	0.000*	413.128	0.000***

***** indicates significant difference from initial value at p<0.001**

Concerning the length, it is positively correlated to the number of new leaves. Autumn gave the longest cutting followed by summer and spring. The longest cutting, 5.8cm, developed during

November, two months after the introduction of cuttings under tunnels. The mean length during the season was 4.4cm (Graph 3; Figure 7).

Figure 7. Cuttings proliferation during september 2010



Similarly to the number of newly developed leaves, cultivar, year, season and days after initiation influenced significantly the length of developed cuttings (Table 4).

The best length was observed on Soury cultivar, during autumn season, 60 days after their introduction in the nursery.

Table 4 . Effect of cultivar, year, season and number of days after initiation on the length of the cutting.

	Cultivar		Year		Season		Days after initiation	
	F	p	F	p	F	p	F	p
Length	35.361	0.000***	438.003	0.000***	59.055	0.000*	502.769	0.000***

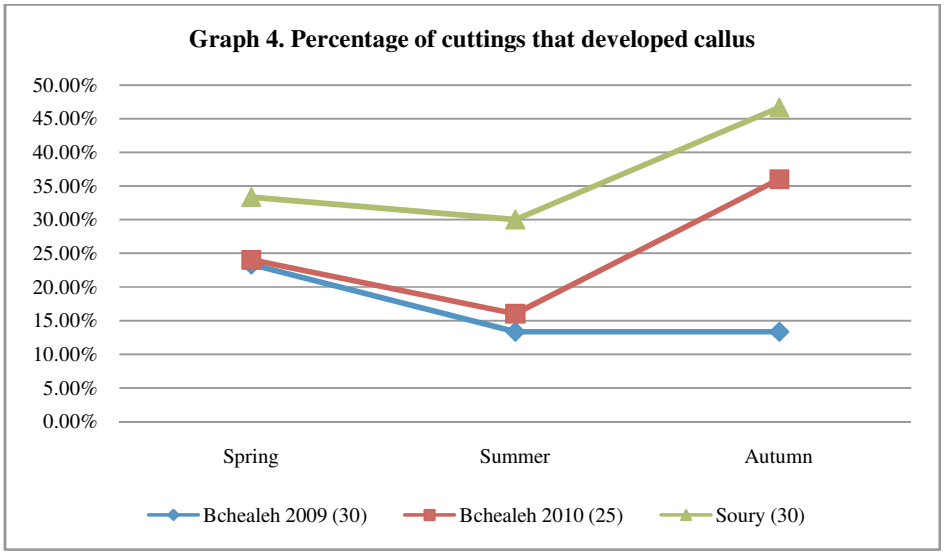
***** indicates significant difference from initial value at p<0.001**

were a very low percentage of Bchealehs cuttings that developed callus (2.6% during 2009 and 5.33% during 2010) one month after the initiation phase; while this number increased to reach respectively 18.5 and 26.5 in 2009 and 2010 in Soury cv. The highest number of developed callus appeared sixty days of the initiation with 27.7 and 38.7%.

During 2009, the callus formations were between 13.33 and 23.33%, while this rate increased to reach 36% during 2010. The highest callus development was during autumn 2010. The lowest percentage of callus formation was during summer season of 2009 and 2010 and this on Bchealehs and Soury cv.

Figure 8. Callus formation on a cutting introduced on November 2010

Compared to Bchealehs cuttings, the ones of Soury cv. had higher percentage of developed callus during the three seasons of collect, spring, summer and autumn with respectively 33.33, 30 and 46.66%. The lowest percentage appeared during autumn 2009 on Bchealehs cuttings with 13.33% (Graph 4).



The number of days after initiation played a crucial role and influenced significantly the development of callus. Concerning the influence of cultivar, year and season, cuttings acted similarly and had the same trend; while, the number of days after initiation influenced significantly the callus formation (Table 5). The best callus formation (32%) was during autumn, followed respectively by spring (26.9%) and summer (19.8%) independently of cultivar.

Table 5. Effect of cultivar, year, season and number of days after initiation on callus formation.

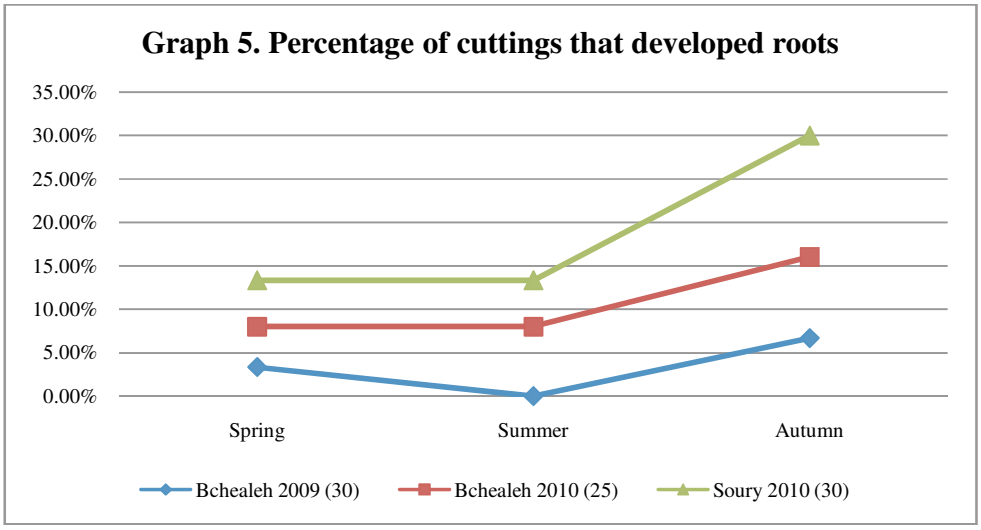
	Cultivar		**Year**		**Season**		**Days after initiation**	
	F	p	F	p	F	p	F	p
Callus formation	1.747	0.187	3.546	0.600	0.487	0.615	32.428	0.000***

***** indicates significant difference from initial value at p<0.001**

The rate of the rooted Bchealehs cuttings varied between 0 and 16.66%. Soury had the highest number of rooted cuttings with 30% followed by Bchealehs cuttings during autumn 2010 (16.66%) (Graph 5; Figure 9).

The low percentage is probably due to the media used that had a high water holding capacity and didn't form a suitable environment for the development of adventitious roots. Isfendiyaroglu *et al*. (2009) got better results when media having a reduced water retention and good aeration were used.

Figure 9. Cuttings with developed root system



The roots formation in Bchealehs cuttings was significantly influenced by number of days after initiation and by the year where the highest number of rooted plants appeared during 2010 in both the Soury cv. and Bchealeh cv. (Table 6; Figure 10).

There was no influence of the season on the rooting and cuttings acted similarly and gave the highest amount of rooted cuttings during autumn followed by spring and summer (Table 6). These results contradicts Loach (1988) who described the negative effect of peat on the root due to its high water retaining capacity especially in autumn where the solar radiation levels are low.

Table 6. Effect of cultivar, year, season and number of days after initiation on roots formation.

	Cultivar		**Year**		**Season**		**Days after initiation**	
	F	p	F	p	F	p	F	p
Roots formation	0.841	0.360	8.864	0.003**	2.952	0.530	23.582	0.000***

**** indicates significant difference from initial value at p<0.01; *** indicates significant difference from initial value at p<0.001**

Canozer and Ozahci (1992), who conducted a three years experiment on the rooting ability of 83 Turkish olive cultivars, observed significant differences between years in terms of root production; while the rooting ability between years have been ascribed to the alternate bearing trend shown by mature stock plants which might be related to the endogenous carbohydrate status (Ozkaya and Celik, 1999).

Figure 10. Rooted Bchealehs cutting eight weeks after its introduction *in vivo*

During acclimatization, and in order to get the highest survival rate, the shades were partially removed in order to reach the optimized humidity for rooted plants.

The increase in cortex thickness during rooting (Ayoub, 2001) could be the main factor that influenced the rooting of Bchealehs cuttings by forming a mechanical barrier for the emergence of the initial roots.

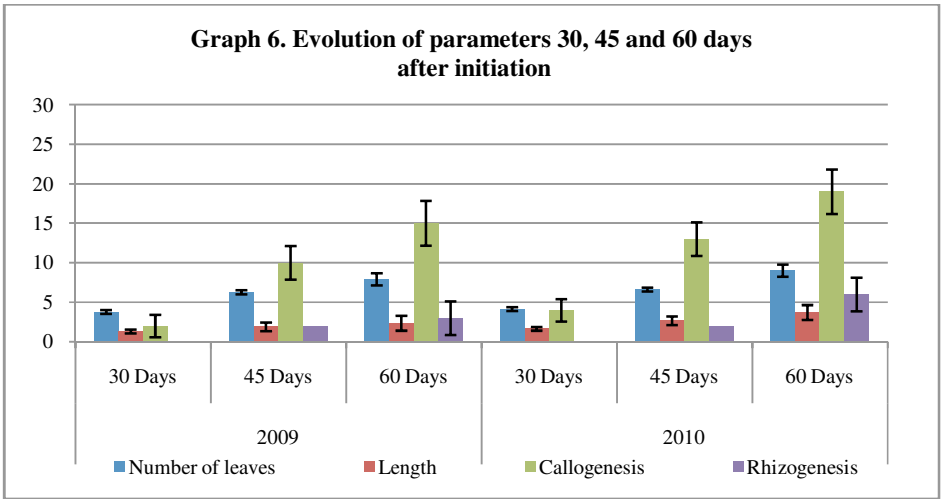
Some cuttings that remained alive didn't develop callus and roots; it could be due to the media used (Peat, Potting soil). Gerakakis and Ozkaya (2005) obtained also cuttings with no roots and callus on other media combination (Perlite, Peat, Sand, Silt), while Ozkaya and Celik (1993) got a successful rooting for difficult to root cultivars on the same combination. Another combination of potting soil and styromousse (1:1) gave the highest percentage of rooted Picual cuttings (92.5%) (Abousalim and Mansouri, 1991). The difference between media could be attributed to the low water retention capacity, very good drainage and aeration of roots.

A very strong positive correlation was observed between the number of newly developed leaves and the length of cuttings (0.911) (Table 7).

Table 7. Correlation between cuttings parameters.

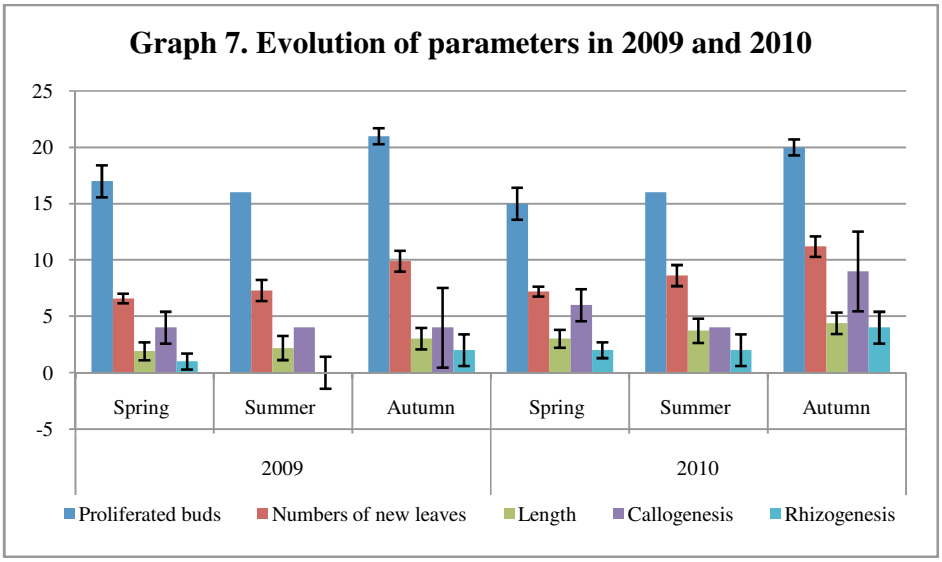
Correlation	Number of newly developed leaves	Length	Callogenesis	Rhizogenesis
Number of newly developed leaves	1	0.911	0.354	0.36
Length	0.911	1	0.391	0.391
Callogenesis	0.354	0.391	1	0.491
Rhizogenesis	0.36	0.391	0.491	1

The number of days after the introduction of cuttings influenced significantly all the parameters: number of new leaves, length, callogenesis and rhizogenesis. During 2010, best results were registered sixty days after the introduction of cuttings with respectively 9 as number of new leaves and 3.7cm of length; while the reported callus formation was 25.33% (19 cuttings). Finally the number of rooted explants was 8% (6 cuttings) (Graph 6).



The years, 2009 and 2010, had the same trend and influenced significantly all the parameters: number of new leaves, length, callogenesis and rhizogenesis (Graph 7).

The mean values of new leaves (11.2), length (4.39), callus formation (36%) and roots development (16%) appeared during autumn 2010, while the best season for the proliferated buds was the autumn season of 2009 with 84%.



The use of NAA on Bchealehs cuttings didn't give the same results as Fernandez *et al*. (2002), who showed that the use of 0.2% NAA powder was more effective on difficult to root "Galega

Vulgar" than treatment with 5000ppm liquid IBA, while Celik *et al.* (1993) observed that a quick dip of "Gemlik", an easily rooted cultivar, in an NAA solution of 2000ppm was more effective than an equal concentration of IBA.

4.2. *In vitro* culture.

The shoots were collected in the morning over three seasons (spring, summer and autumn) (Figure 11). Then disinfection, by a mix of fungicides and bactericides before the initiation of axillary buds *in vitro*, was applied.

The first step consisted on disinfecting the plant material by a fungicide mix composed of propamocarb-HCl, carbendazim and fosethyl-aluminium for two hours, followed by a bactericide treatment composed of NaOCl (15%) and Chloroxylenol (2.5%) in the laboratory. This sterilization method gave the most satisfactory results especially when fungi cause serious problem in the initiation phase. It is to note that media were added by 1mg/l of oxytetracycline in order to minimize the percentage of the browning tissues and bacterial contamination during the initiation phase.

Figure 11. Shoot before its disinfection and introduction *in vitro*

4.2.1. Initiation phase.

The observations, five weeks after the introduction of explants *in vitro*, focused on of the percentage of the proliferated buds, number of new explants, and number of explants lost by fungal, bacterial and browning tissues.

The initiation phase constitutes the most critical phase in the adaption of plant material to the first stage *in vitro* where the balance auxin/cytokinin plays an important role in buds proliferation (Zuccherelli and Zuccherelli, 2003). A total of 810 cuttings were introduced on MS media (Murashige and Skoog, 1962), OM media (Ruggini, 1984) and WPM. These media were supplemented with BAP at different concentrations (2, 3 and 4mg /l). One week after its introduction, the buds started to proliferate (Figure 12).

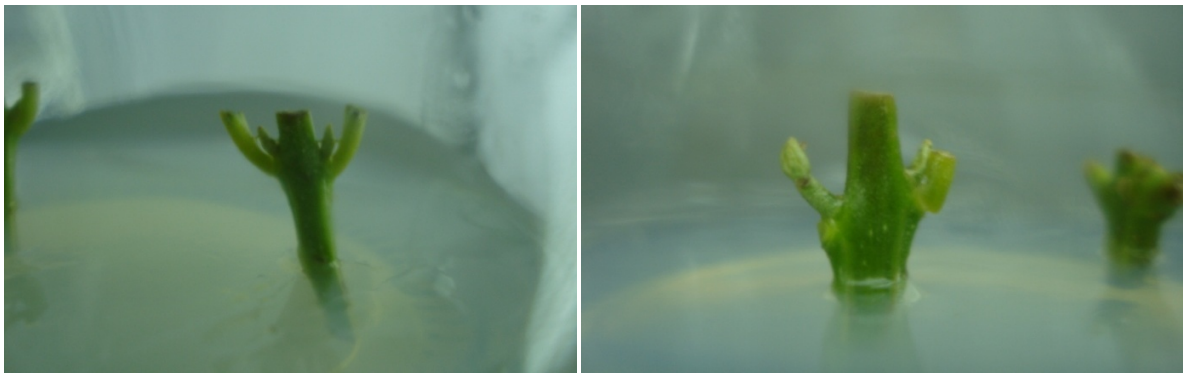


Figure 12. *In vitro* emergence of axillary buds during the initiation phase

Even when the mix of fungicides and bactericides was used, bacterial contamination (12%), fungal (13.3%) continued to appear. While the browning tissues resulted respectively in loss of 23 and 29% of the material introduced during 2010 and 2009 (Figure 13). The same problem was observed by Grigoriadou and Vasilakakis (2000) on the leaves and buds of Chalkidikis cv. causing losses in the plant material. This oxidation may be due to the sensitivity of explants to the treatment used during this phase or the size of axillary buds introduced being less than 0.5cm (Figure 13).

The season influenced significantly the newly developed explants and the highest values appeared during the two autumns of 2009 and 2010 with respectively 1.35 and 1.33, while only 1.12 new explants developed during summer of 2009 (Table 8). The percentage of proliferated buds acted similarly in the three seasons with best survival rate observed during autumn 2009 (47.1%) followed by summer 2009 (46.2%) while the lowest rate was during 2010 (31.1%).

Concerning the browning tissues, it was highly influenced by the season and the rate ranged respectively between 23.7 (summer 2009) and 34.1 (spring 2009) (Graph 8). The oxidation results are in accordance to Ramzan Khan *et al.*, (2002) where explants from adult cuttings suffered from rapid oxidation despite the preventive treatments by adding ascorbic acid and citric acid.

For the bacterial contamination, the highest amount was observed during autumn 2010 (22.6). Concerning the number of explants lost by fungal contamination, it was not influenced by the year. The difference between the two varieties has remained below 13.5% (Table 8; Graph 8).

Figure 13. Browning explant during the initiation phase

Our results are in accordance with those obtained by Bougdal *et al*. (2007) who had the best rate of proliferated buds (90%) during autumn, followed respectively by spring (40%), summer (16%) and winter season (0%). According to Abousalim *et al*., (1993), the active seasons of olive growth are spring and autumn. Plant material introduced during spring will give explants with low vegetative growth due; this could be due to the flowering effect on the tree.

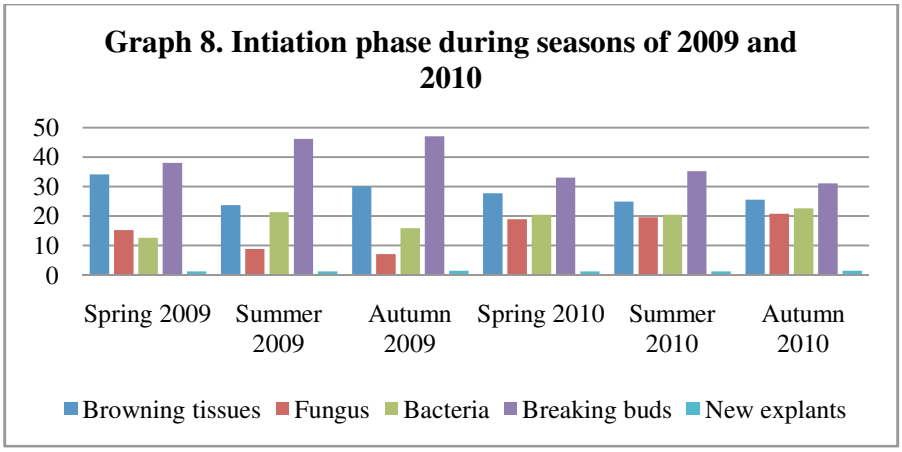
These results are similar to ones observed on the two Lebanese cultivars, Baladi cv. and Chatawi cv., which showed the best proliferation during autumn with 61%, followed by spring 37.5% and summer 24.5% (Boustany, 2008). A full blossom and fruit set, during spring and summer, could

influence negatively the regeneration of the explants introduced *in vitro*; this is why, and during autumn the highest percentage of proliferated buds was observed.

Table 8. Influence of the season on the percentage of the proliferated buds, newly developed explants, browning tissues, bacterial and fungal contamination during the initiation phase.

	Season	
	F	p
Number of proliferated buds	12.771	0.073
Number of newly developed explants	74.462	0.013*
Number of browning tissues	23.153	0.000***
Number of explants lost by bacterial contamination	130.435	0.008**
Number of explants lost by fungal contamination	15.198	0.062

*** indicates significant difference from initial value at p<0.05; ** indicates significant difference from initial value at p<0.01; *** indicates significant difference from initial value at p<0.001.**



The effect of the year was also studied and didn't show any significant influence on the studied parameters (Table 9). For the percentage of broken buds, the highest rate (43.7%) was obtained during 2009, while the new developed explants acted similarly in 2009 and 2010 and gave 1.22 as a mean value. Concerning the browning tissues, 2010 was better than 2009 and had the lowest rate with 26% (Graph 9).

The bacterial and fungal contaminations were both lower in 2009 with respectively with 10.4 and 16.6%. These losses are still lower than ones observed on Leccino cv., where the culture

contamination was between 50 and 60% even when $HgCl_2$ (0.1%) with NaOCl (50%) were used (Ramzan Khan *et al*., 2002).

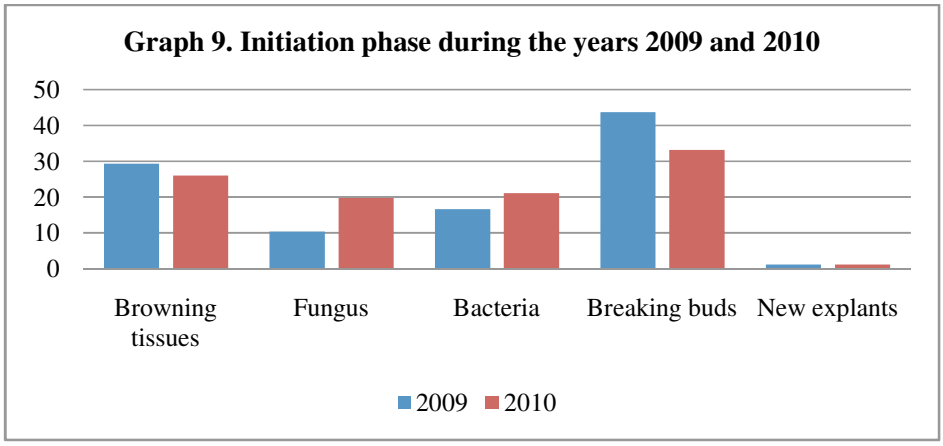
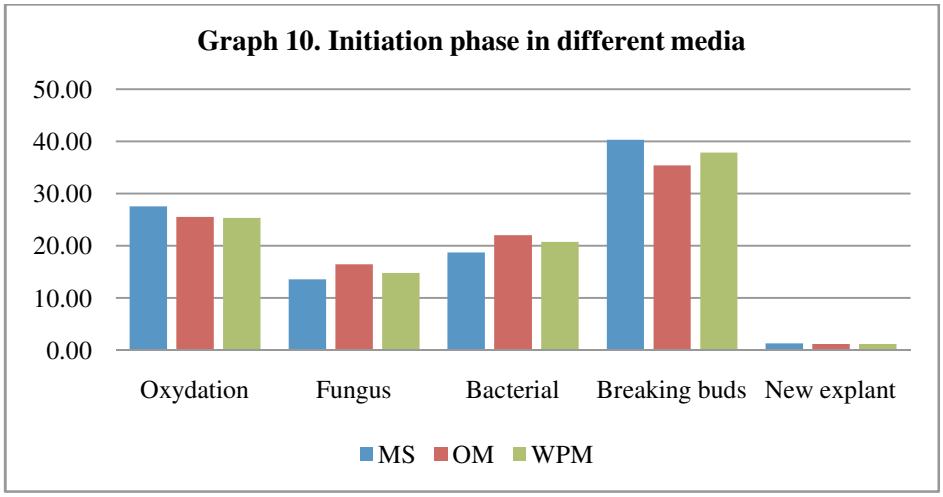


Table 9. Influence of the year on the percentage of the proliferated buds, newly developed explants, browning tissues, bacterial and fungal contamination during the initiation phase.

	Year	
	F	p
Number of proliferated buds	0.021	0.898
Number of newly developed explants	0.021	0.898
Number of browning tissues	0.007	0.934
Number of explants lost by bacterial contamination	0.021	0.898
Number of explants lost by fungal contamination	0.070	9.390

The influence of media, with the exception of the browning tissues, didn't influence the other parameters. The highest rate of browning tissues appeared on MS media with 27.6% (Graph 10). The components of media couldn't have any influence on the mycelium development of fungus nor do bacterial colonies (Table 10). Nor the media, nor the added hormone had influenced the percentage of proliferated buds and new explants. The new explants and percentage of broken buds varied between 1.17-1.19 and 35.4 and 40.3% respectively.

These results are similar to the ones observed in a previous study on two Lebanese cultivars Baladi cv. and Chatawi cv., initiated on twenty three media, where no significant difference was observed and newly introduced explants acted similarly during the initiation phase (Boustany, 2008).



Even so media hadn't any influence on the new explants and breaking buds, results showed that during 2009, MS media was the best for the development of new explants with 1.46 on MS added with 3mg/l BAP during 2009 while the highest percentage of breaking buds was on MS added with 2 or 3mg/l who gave 60% during autumn and summer 2009. During 2010, MS added with 3mg/l BAP during autumn gave the highest rate of newly developed explants, while WPM added with 2mg/l BAP had the highest breaking buds.

Our results are in accordance with those obtained on the Algerian Chemlal cv. where the best axillary bud proliferation was obtained from autumnal explants introduced on MS media added with 2mg/l BAP or 2mg/l Kinetin with respectively 75 and 90% (Bougdal *et al*., 2007). Media, OM and MS/2, were the most effective on Moroccan Picholine cv. during the initiation phase and proliferated buds exceeded 90% (92 and 91%) (Brhadda *et al*., 2003).

Media MS was better than OM for three Syrian varieties, Dan, Sorani and Zeiti. In Greece, Chondrolia chalikidikis cv. proliferated better on WPM than OM and QL (Quoirin and Lepoivre) media, while in the Meski cv., the best result was recorded on MS media when compared to OM and P media.

Our results differ from the ones observed on Nebbiara cv. that proliferated better and gave longest shoots on OM than MS (Zacchini and De Agazio, 2004). The BAP (1mg/l) added to

OM/2, was sufficient to have the highest rate of bud break in the varieties Arbequina and Picual (Garcia-Ferriz *et al.,* 2003).

Abousalim *et al*. (2004), achieved the highest rate of bud break (100%) and development of new shoots with 5mg/l Zeatin OM on the environment. The medium OM/2, including macronutrients diluted by half and supplemented with Zeatin (4 mg / l) was most effective for axillary bud with rates close to 80% for the variety and Aglandou Tench (Binet *et al*., 2006).

Table 10. Influence of the media on the percentage of the proliferated buds, newly developed explants, browning tissues, bacterial and fungal contamination during the initiation phase.

	Media	
	F	p
Number of proliferated buds	6.255	0.145
Number of newly developed explants	9.351	0.100
Number of browning tissues	29.460	0.033*
Number of explants lost by bacterial contamination	1.071	0.400
Number of explants lost by fungal contamination	1.794	0.407

*** indicates significant difference from initial value at p<0.05**

Four weeks after their introduction, die back symptoms appeared and were followed by apical buds detachment. This problem proliferated on different media. This decline, observed only during spring and summer; it started at the end of the proliferation and then spread to the starting explant causing its loss (Figure 14). This problem, also observed on a previous study on two Lebanese cv., Baladi and Chatawi cv. appeared during spring, caused the loss of 23% of explants.

Figure 14. Die back of the explant during the initiation phase

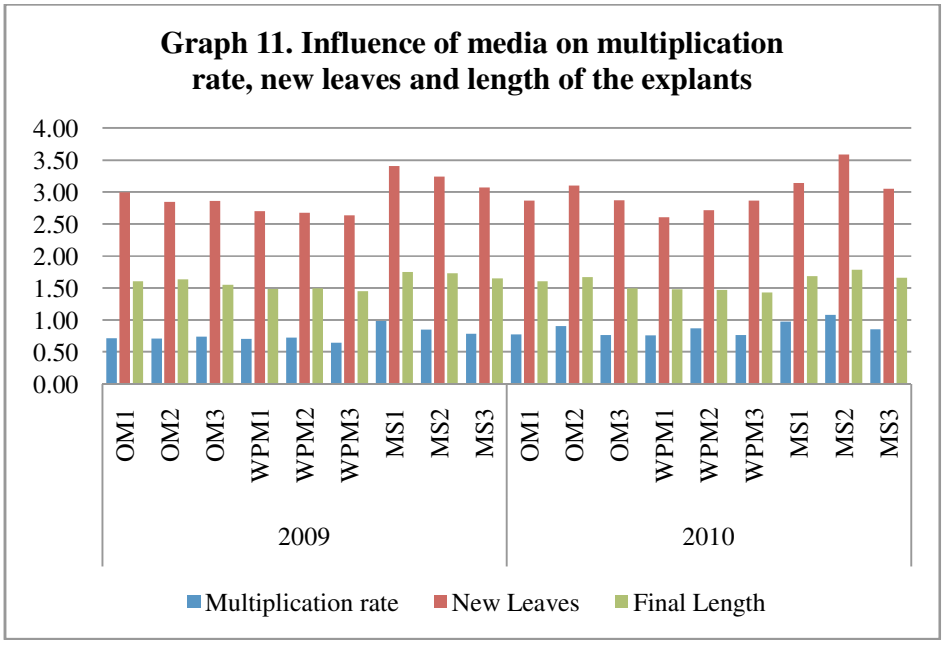
4.2.2. Multiplication phase

Five weeks after their introduction, explants were ready to be transplanted and formed the first subculture (Figure 15). The plant material was subcultured for three times before the transfer of the plant material on elongation medium.

Figure 15. Cutting at the end of the initiation phase

Once explants were subcultured, a medium effect was observed and showed the superiority of the medium MS added with 2 and 3 mg / l BAP. The multiplication rate was also influenced significantly and the highest number of new explant (1.08) was on the MS2 followed by MS1 with 0.98 (Table 11; Graph 11). For the new leaves and length of explant, the same media (MS2 and MS1) gave the highest amounts with respectively 3.58 and 3.41 newly developed leaves and 1.78 and 1.75 cm.

The lowest multiplication rate and the shortest explants were observed on the medium WPM3 (WPM added with 4mg/l BAP) with an average of 0.64 new explant and 1.43cm, while the lowest number of new leaves were observed on WPM2 (WPM added with 3mg/l BAP) (Graph 11).



The MS media showed their superiority during 2009 and 2010 on the studied parameters followed by the OM (Ruggini, 1984). The lowest rates were observed on the WPM media and this during the two years of experiments.

These results contradicts the results recorded on cv. Moraiolo, where OM proved its superiority on WPM and produced the highest number of shoots per proliferated explant (0.84), which was significantly higher than that on WPM medium with 0.51 new explant (Ansar *et al.*, 2009).

Concerning the concentration of the growth regulator (BAP), the highest quantity used (4mg/l) influenced negatively the multiplication rate that decreased with subculture. This result is in accordance with Ansar *et al.*, (2004) who showed that high concentration of growth regulators significantly reduced shoot multiplication on OM and WPM. The shoot height in Leccino cv. was reduced with the increase in the concentration levels of cytokinins (BAP, 2ip and Kinetin) (Ramzan Khan *et al*., 2002).

Dimassi-Theriou (1994) reported that BAP alone was more effective than 2ip for the shoot proliferation and when added at a concentration of 5 to 7.5 ml/l, BAP gave 1.2-1.8 shoots/explant and didn't influence the height of Kalamon olive (Rama and Pontikis, 1990).

The results concerning the length of Bchealehs explants, where MS medium was the best followed respectively by OM and WPM, are in accordance with those observed on Moraiolo cv. in which OM provided the best shoot length (2.25cm) when compared to WPM (1.24cm).

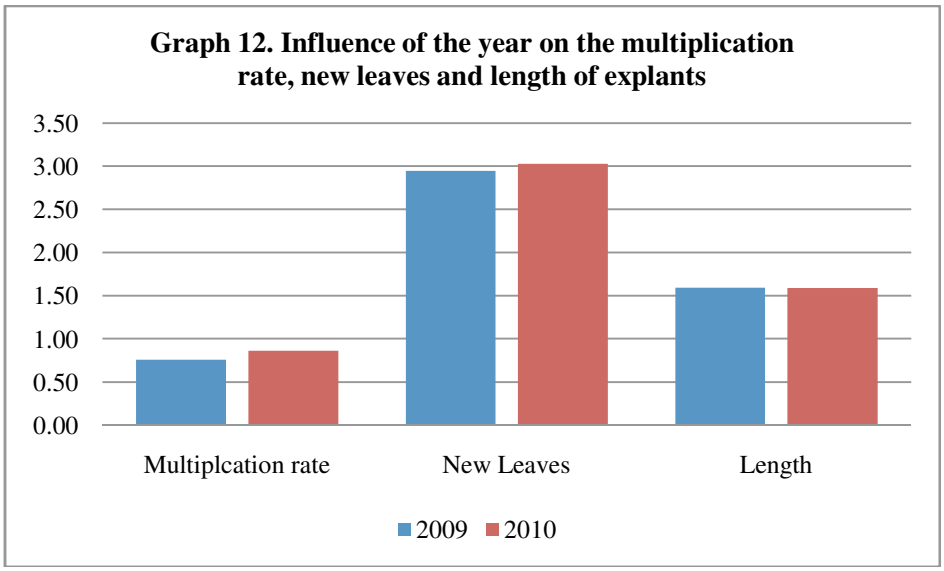
Table 11. Influence of the media on the multiplication rate, number of newly developed leaves and length of the explant during the multiplication phase.

	Media	
	F	p
Multiplication rate	6.456	0.000***
Number of newly developed leaves	3.257	0.002**
Length of the explant	6.834	0.000***

**** indicates significant difference from initial value at p<0.01; *** indicates significant difference from initial value at p<0.001**

In Moroccan Picholine cv., media influenced significantly the length of developed shoots and this after two subcultures; it was observed that OM gave the longest shoots (1.24cm), followed by MS/2 (0.89cm) and WPM (0.3cm) (Brhadda *et al.*, 2003).

The year had a significant effect on the multiplication rate of Bchealehs explants and this may be due to the application of foliar fertilizers during the season 2009 that enhanced the vegetative growth and gave well developed twigs on the tree independently of the seasons of 2010 (Table 12).



The best multiplication rate was observed during 2010 with 0.86 instead of 0.76 during the previous year (13% increases), while newly developed leaves acted similarly and didn't show any important modification (2.7%) and passed from 2.95 to 3.03; the mean values of the new explants length was the same during 2009 and 2010 and recorded 1.59 (Graph 12).

Table 12. Influence of the year on the multiplication rate, number of newly developed leaves and length of the explant during the multiplication phase.

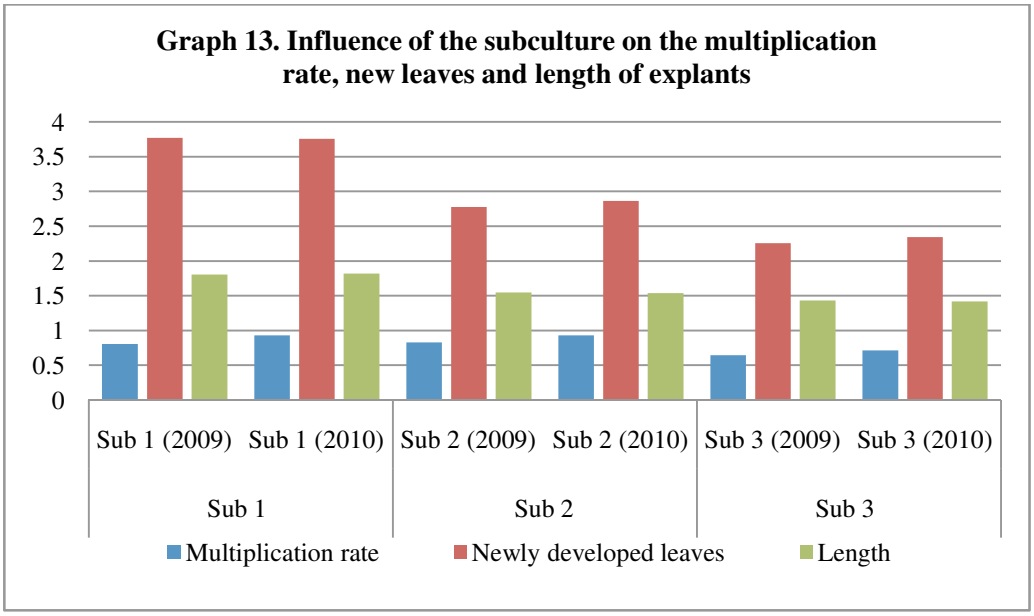
	Year	
	F	p
Multiplication rate	14.028	0.000***
Number of newly developed leaves	0.918	0.339
Length of the explant	0.682	0.410

***** indicates significant difference from initial value at p<0.001**

A subculture effect was observed and influenced significantly the multiplication rate, the number of new leaves and length of explants during the three seasons of the two years (Table 13). The first subculture gave the highest rates followed respectively by the second and the third subculture (Graph 13).

Figure 16. Explant at the end of the first subculture during 2010

The highest multiplication rate (0.93) was recorded during the first subculture of 2010 followed by the second subculture of the same year with (0.925) and decreased during the third to 0.71 (Graph 13, Figure 17).



The highest number of newly developed leaves was during the first subculture (2009) with 3.77, while the lowest (2.26) was recorded during the third subculture of the same year (Graph 13).

Figure 17. Explant at the end of the second subculture

Table 13. Influence of the subculture on the multiplication rate, number of newly developed leaves and length of the explant during the multiplication phase.

	Subculture	
	F	p
Multiplication rate	25.966	0.000***
Number of newly developed leaves	118.176	0.000***
Length of the explant	52.341	0.000***

***** indicates significant difference from initial value at p<0.001**

The length decreased from subculture to another and passed from 1.45 to 1.14cm during 2010, and from 1.82 to 1.42cm during 2009 (Figure 18).

Figure 18. Explant of the third subculture

The season played an important role and influenced significantly all the studied parameters, especially the length of the explant due to the vigorous shoots introduced *in vitro* after the fertilizer applications (Table 14). The highest number of new explants (0.8 and 0.94) was observed during autumn, followed by spring season and summer. The lowest number was recorded during summer with respectively 0.71 and 0.79 during 2009 and 2010 (Graph 14).

Concerning the length, the shortest explant was the one developed during summer (1.48cm), while the longest was observed during autumn with 1.69cm. For the newly developed leaves the highest number (3.35) observed during autumn 2010 while the lowest appeared during spring of the same year with 2.78 (Graph 14).

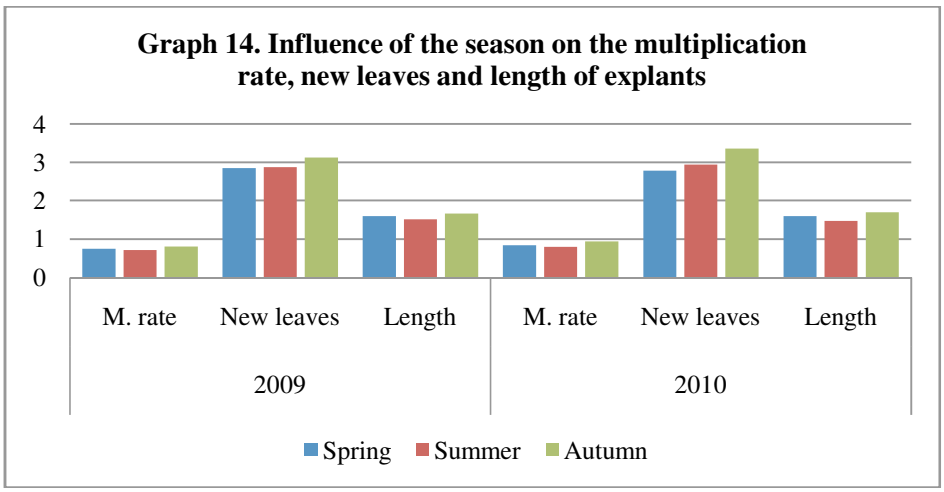


Table 14. Influence of the season on the multiplication rate, number of newly developed leaves and length of the explant during the multiplication phase.

	Season	
	F	p
Multiplication rate	5.364	0.006**
Number of newly developed leaves	4.528	0.012*
Length of the explant	9.641	0.000***

*** indicates significant difference from initial value at p<0.05; * indicates significant difference from initial value at p<0.01; * indicates significant difference from initial value at p<0.001.**

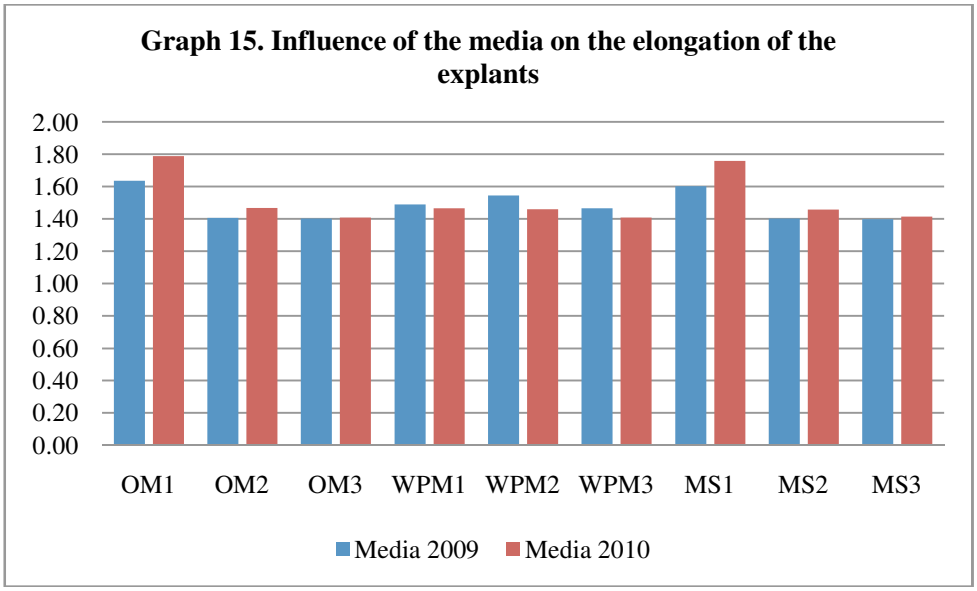
4.2.3. Elongation phase.

Due to the decrease in the length of the subcultured explants and the loss by oxidation, all the explants were transplanted on media free of hormones and this for five weeks before their exposition to media added by NAA and IAA to enhance rooting.

The best recorded elongation was on the explants issued from the OM1 followed by the MS1 and this for the two years with respectively 1.79 and 1.76 during 2010; 1.64 and 1.6cm during 2009 (Figure 19; Graph 15).

Figure 19. Elongated explant on MS medium

All the remaining explants couldn't exceed 1.5cm and ranged between 1.4 and 1.47cm with one exception the WPM2 (1.54cm) during 2009 (Graph 15).



4.2.4. Rooting phase.

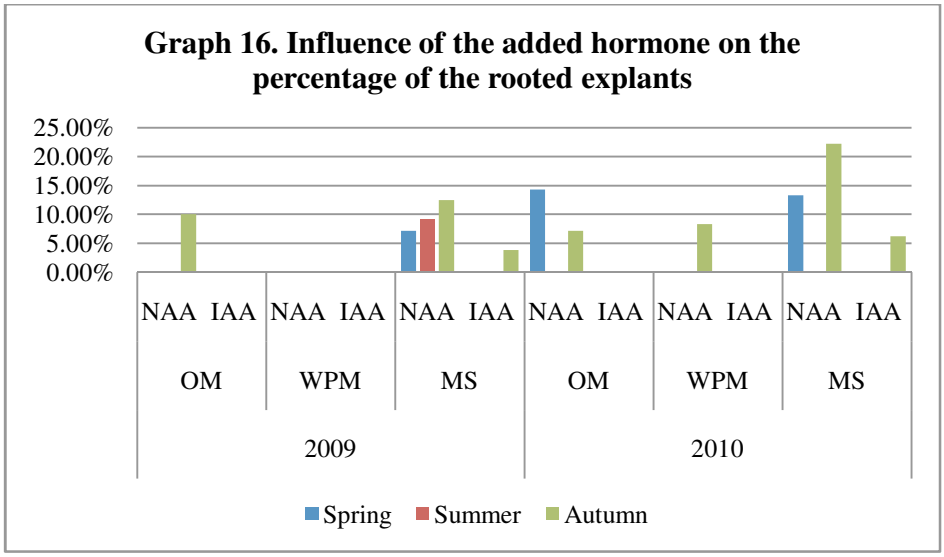
After the elongation phase, all the explants were transferred to fresh media added with 2mg/l of NAA or IAA. A significant difference was observed and showed the superiority of the MS media added with NAA that gave the highest amounts of rooted explants (22.22% of the explants issued from the elongation phase) followed by OM media added with NAA and gave 14.3% of rooted explants (Graph 16). Ruggini (1984) reported that NAA at 1mg/l is the most efficient for the rooting of explants issued from water sprouts, yielding branches and non productive shoots.

Abousalim *et al*. (2005) observed that plant material issued from adult olives didn't develop any root system even when different concentrations of auxins were added while the juvenile material developed callus at the base of explants (97%) and rooted at 2mg/l of NAA (94.4%).

The rooted explant on WPM medium had chlorotic leaves that showed similar symptoms of calcium deficiency (Figure 20). This may be due to the high concentration of chloride in the medium; this is why the rooted explant was transferred on MS medium in order to minimize the effect of chloride on the rooted explant.

Figure 20. Rooted explant with chlorotic leaves on WPM medium added with 2mg/l BAP

Season also influenced the percentage of rooted plants. Explants introduced during autumn showed the highest capacity of rooting followed by spring season. In total, 4.3% (16 over 374) of explant rooted (Figure 21).



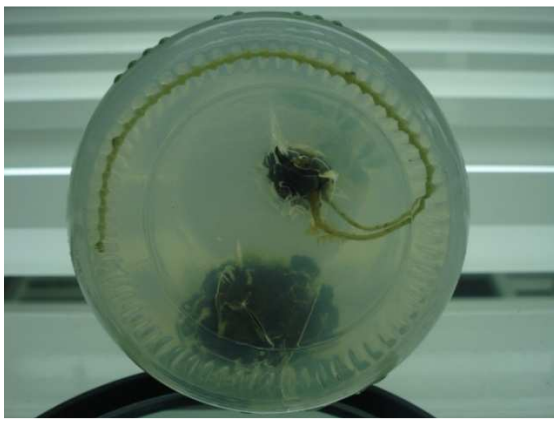


Figure 21. Explant with developed root system on MS medium added with 2mg/l of BAP and ready to be transferred to the nursery to be acclimatized

4.2.5. Callogenesis.

During autumn 2009, and three weeks after their introduction, explants started to develop massive calluses. This is why and during the three seasons of 2010, plant material was introduced *in vitro* to study the effect of the seasons on the callus formation. Twenty explants were cultured on same media.

None of the introduced explants during spring and summer developed callus. Only explants introduced during autumn developed callus. Media didn't influence the development of callus and appeared almost in the same amounts, the rate ranged from 26.7 to 33.3%; the 26 explants were transferred on a fresh media where they were conserved in the same conditions and subjected to ten days dark (Figure 22).

The development of callus on Bchealehs explants is in agreement with the findings of Ruggini (1984), who showed that Frantoio, a difficult to root cultivar and less suitable for *in vitro* culture produced abundant basal callus, while Moraiolo, an easy to root cultivar had the fastest growth rate. Increased concentration of BAP (4 and 8mg/l) enhanced callusing at the base portion of shoots (Ramzan Khan *et al.*, 2002).

Figure 22. Callus formation at the end of explants introduced during September 2009

Three weeks later, roots started to emerge from the base of the explant. The rooted explants were then transferred to a media added with 2mg/l of NAA to induce the root development. At the end, 6 over the 26 explants developed roots (23.1%) (Figure 23).

The introduction of olives explants during autumn could be the fastest technique to ensure rooted explants without passing by any subculture especially that this cultivar showed difficulties in rooting.

Figure 23. Root emergence from developed callus during October 2009 (Callus were transferred to a fresh media)

4.3. Olive fruit parameters.

4.3.1. Weight of olive drupes.

The fruit weight is considered an important measure to determine olive fruit quality for table olive cultivars (Al-Hazmi, 2007). The average weight of olive drupes was significantly influenced by the ripening process and crop year (Table 15; Figure 24). The maximum weight was reached two weeks before the end of the ripening process in 2009 and 2010 with respectively 3.84 and 4.31g (Graph 17). The increase in fruit size is due to a greater development of epicarp (Barone *et al*., 1994). This difference may be due to the negative relationship between the fruit weight and number of fruit on the trees, which is attributed to the high crop load of trees during the season 2009. Similar results were obtained by Al-Maaitah *et al.* (2009). The average fruit weight fall in the average fruit weight of olive cultivars in the world (Al-Hazmi, 2007).

The highest increase was observed between the first and the second collect, where 17% of fruit growth was noticed (Graph 17). The slower increase observed later between different collects could be a result of the decrease of the auxin level in the fruit or the competition on the auxin between the embryo and fruit flesh tissue, as a result the enlargement of the flesh is also slow (Desouky *et al*., 2010).

Figure 24. The green skin changing gradually to become totally black and over ripe (season 2009).

The first five collects had a maturity index that didn't exceed 1.3, while starting the sixth collect, the maturity index passed from 2.45 to reach 6.5 when drupes started to over ripe.

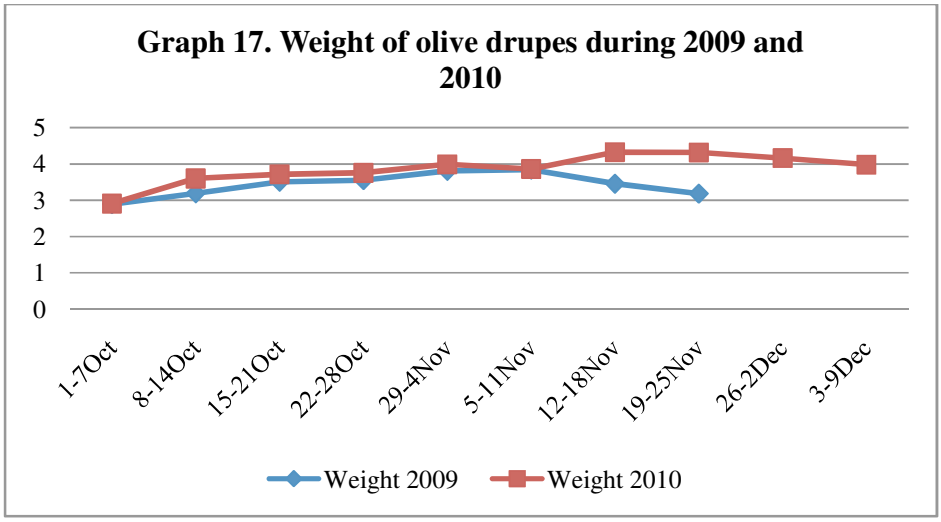
When over ripped, the drupes started to lose weight and a decrease was observed (Graph 17). Exogenous factors such high temperature, non available moisture or severe evaporation may decrease the growth rate of the fruit (Desouky *et al*., 2009). The increase in the fruit weight, between the fourth and the seventh collect, and during the two years of 2009 and 2010, didn't exceed 6.6%.

Table 15. Effect of the year, collection date and repetition on the olives weight.

	Year		Collection date		Repetition	
	F	p	F	P	F	p
Weight	17.189	0.001**	59.056	0.000***	1.443	0.214

**** indicates significant difference from initial value at p<0.01; *** indicates significant difference from initial value at p<0.001**

Respectively during 2009 and 2010, around 10 and 4.55% of the weight were lost within one week after the over ripe of the drupes (Graph 17). The increase of fruit size during the last phase comes mainly from the increase of moisture content of the fruit (Desouky *et al*., 2009) and the oil accumulation; that's why, the mean value of the moisture content of olive drupes within the same period acted similarly as the fruit weight with a difference of only 3.35%.



4.3.2. Width of olive drupes.

The width showed high significant differences within the season, and the crop year (Table 16). For the year 2009, it ranged between 1.41 and 1.58 cm; while during 2010, the width ranged between 1.52 and 1.79cm (Graph 18).

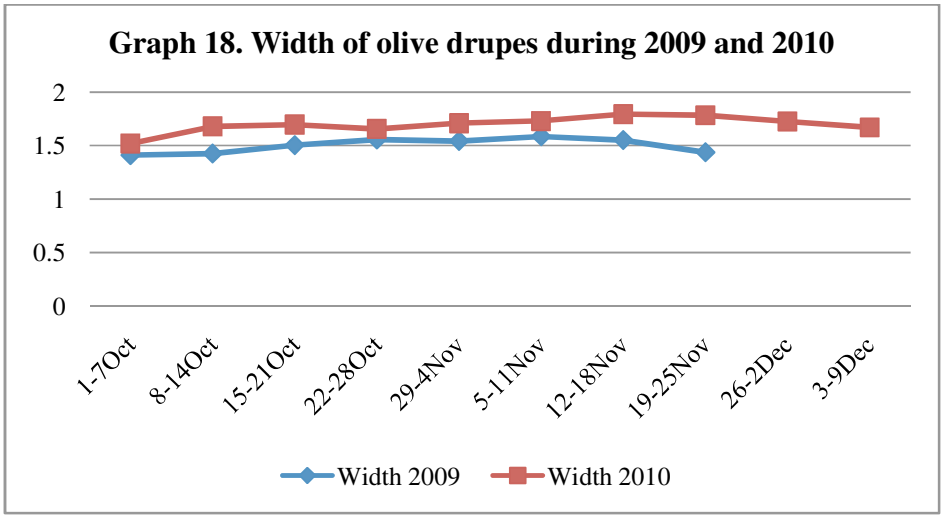
Table 16. Effect of the year, collection date and repetition on the olives width.

	Year		Collection date		Repetition	
	F	p	F	p	F	p
Width	303.08	0.000***	34.701	0.000***	1.556	0.212

***** indicates significant difference from initial value at p<0.001**

The mean of maximum diameter (1.58cm) was observed on the sixth collect and then decreased to 1.44cm at the end of the ripening process during 2009. The same behavior was observed

during 2010, the drupes reached the maximum diameter 1.79 on the seventh collect. The width acted similarly to the weight of olives and had the same trend that started with an increase and one week after the color change from purple to black, a slight decrease was recorded (Graph 18).



The differences in width of olive drupes between 2009 and 2010 are mainly due to the high yield of 2009.

4.3.3. Pit weight and Flesh/Pit ratio.

A large variation in fresh weight within the Bchealehs drupes was observed during ripening, while the weight of the pit didn't vary significantly within the season and the two studied years. The mean values of 2009 and 2010 were respectively 0.63±0.2 and 0.64±0.1g. Results are similar to Tanilgan *et al*., (2007) who found the same seed weight in Uslu variety (0.63g). This stability in the pit weight is due to its early full development in the season, while the flesh continued to develop until reaching its full maturity and then started to decrease.

Our results are in agreement with those obtained by Desouky *et al*. (2009) who found that pit weight values obtained during green, purple and black harvesting stages, had no significant differences during the ripening process. Rahmani *et al.*, (1997) observed an unchanged stone weight during fruit maturity and a constantly increased flesh to pit ratio with increased fruit maturity.

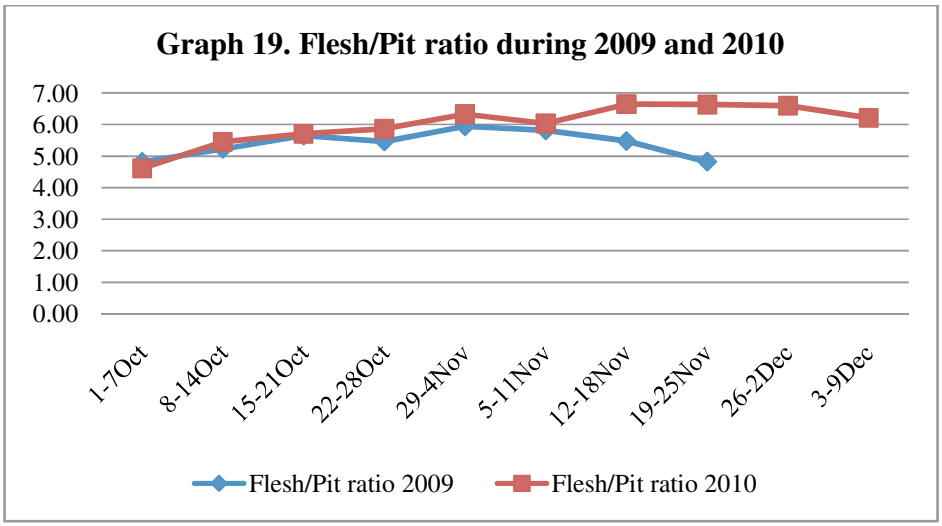
Concerning the Flesh/Pit ratio, it showed a general increase during the drupes ripening and had the same trend of the fruit weight where an increase started from the first collect until reaching full maturity that corresponds respectively, in 2009 and 2010, to the fifth and seventh collect with a value of 5.9:1 and 6.6:1 (Graph 19). This difference is mainly due to the heavy yield during 2009 that influenced significantly the weight of olives drupes (Table 17).

Table 17. Effect of the year, collection date and repetition on the flesh/pit ratio.

	Year		Collection date		Repetition	
	F	p	F	p	F	p
Flesh/Pit ratio	28.543	0.000***	17.103	0.000***	0.972	0.526

***** indicates significant difference from initial value at p<0.001**

Robards and Mailer (2001) obtained at full maturity an average of 5.2:1 as flesh/pit ratio, while the ratio should be at least 5:1 for olives to be acceptable as table olives.



4.3.4. Shape index.

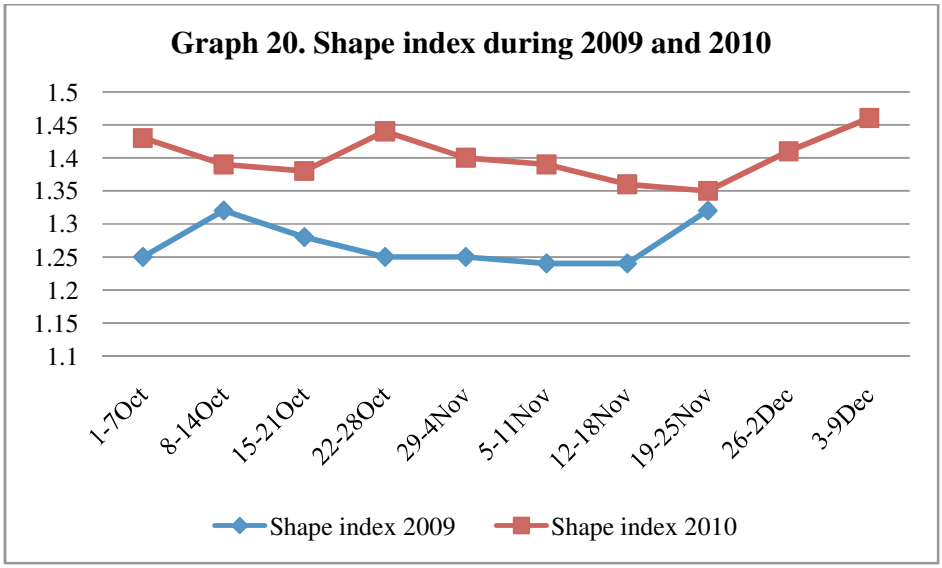
For the shape index that represents the Length/Width ratio, the two years had the same trend and was divided into three phases. The first phase started with an increase followed by a decrease during the fruit development which was due to the continuous growth of drupes in diameter; while the increase during the third phase was due to the over ripped drupes.

Table 18. Effect of the year and collection date on the shape index.

	Year		Collection date	
	F	p	F	p
Shape index	40.476	0.000***	0.616	0.756

***** indicates significant difference from initial value at p<0.001**

The shape index was significantly influenced by the year and ranged within the season between 1.25 and 1.32 during 2009; and 1.38-1.44 during 2010 (Table 18). The highest rate was observed during the fourth collect of 2010 with 1.44 (Graph 20), while the date of collect didn't influence the shape index and increased similarly during the ripening process.



4.3.5. Moisture content.

The moisture content in olive drupes can be influenced by several factors including rainfall, evaporation, soil type, altitude, and tree health.

Moisture content in the olives is important to oil quality for several reasons. First, the moisture drops could cause a cell breakdown that leads to an increase in free fatty acids and therefore lower oil quality. Second, fruit growth, oil synthesis and its accumulation could be limited to environmental stress during mesocarp development. Third, if moisture is very high during the harvest, it may affect the fruit quality and could lead to low yields result from cold press extractions.

Results showed a high significant difference in the moisture content within the season and the production years of 2009 and 2010 (Table 19). The results shown in the graph indicates that the moisture content decreased while the ripening process progressed (Graph 21); this is due to the loss of water due to the cracks on the protective wax around the epicarp or other epidermal openings.

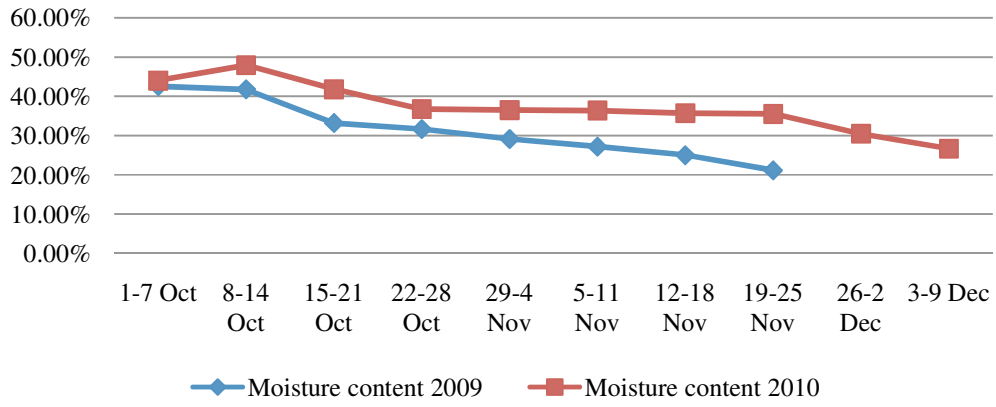
Table 19. Effect of year and collection date on the moisture content.

	Year		Collection date	
	F	p	F	p
Moisture content	751.392	0.002**	81.949	0.000***

**** indicates significant difference from initial value at p<0.01; *** indicates significant difference from initial value at p<0.001**

The slight increase of the moisture of the second collect (47.94%) is due to the rainfall during the first week of October 2010. As shown in the graph, in 2009, the moisture decreased from 42.55% during the first collect to reach 21.25% at the end of the ripening process; while in 2010, this percentage decreases from 44.03% to 26.68% in over ripe drupes (Graph 21).

Graph 21. Moisture content during seasons 2009 and 2010



The results of Bchealehs oil agreed with previous results obtained by Hartmann (1991) and Al-Maaitah (2009). During harvesting, the moisture reduction in fruit could be due to the metabolism process that occurred inside drupes (Cimato, 1990).

4.4. Olive oil parameters.

4.4.1. Oil content.

The oil content was significantly influenced by the ripening process and increased according to increased Maturity index. A significant difference was also observed within the crop year (Table 20). Similar to other olive cultivars, there was a slow and continuous increase in oil content in parallel to the ripening process. Oil begins to accumulate in the fruit in late august and early September (Desouky *et al*., 2009).

The increase in the oil content occurred during the fruit coloration and reached the maximum content when the olive fruit turned into black color respectively at the seventh and sixth collect during 2009 and 2010 with a mean value of 26.46% and 28.96% (Graph 22); there was a decrease trend at the end of the season for the oil content, the oil continued its accumulation until the epicarp blackening and then its content decreased. This decrease, at the end of the season, is due to the degradation of the oil.

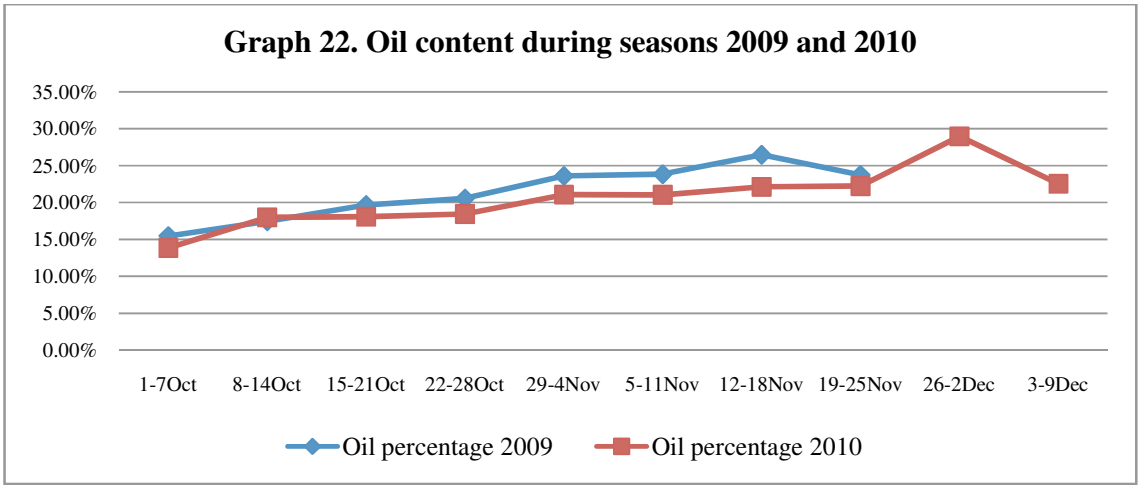
During ripening process, Salvador *et al*. (2001) showed that oil percentage increases dramatically during early fruit ripening and declines slightly as fruit becomes over ripe. In Bchealehs oil, the oil content increased from the first week of October till the third week in November (2009) and first week in December (2010). Hartmann and Opitiz noticed that there was a gradually increase in the oil content during the fall and winter and reaches its maximum as fruits become completely black; while Barone *et al*. (1994) noticed that oil accumulation rate decreased when fruit epicarp became fully black.

Table 20. Effect of year and collection date on the oil content.

	Year		Collection date	
	F	p	F	p
Oil content	81.949	0.000***	16.719	0.000***

***** indicates significant difference from initial value at p<0.001**

According to Lavee and Wodner, the final oil content depends on the interaction between the genetic potential of the variety, growing conditions as well as on the amount of mesocarp available for oil biosynthesis.



The effect of yield on oil percentage could be related to reduced competition between fruits which resulted in a homogeneous crop with large fruits and shorter duration of fruit ripening in trees with light yield. This explain the drop of the oil content in 2009 one week earlier than 2010.

4.4.2. Fatty acids variation.

The fatty acid composition of olive oil, determined by gas chromatography, is an important parameter in the length of shelf life affected by two factors, the olive variety used in the production of the oil and the ripening stage at which the olive is harvested (Beltran *et al*., 2004).

Bchealehs oils have a high content of oleic acid (75.24%) that ranges between 68-86.15%. The seasonal changes in fatty acids composition during the year 2009 and 2010 are shown in the table 21. Table shows that oleic acid clearly dominates. No significant difference was observed and the oleic acid acted similarly during 2009 and 2010 and during all the collects (Table 22).

Romero *et al*., (2003) observed that lower contents of oleic acid and C16:C18 ratio are produced during wet summers while higher contents appears in oils from olives grown under water stress (Salas *et al*., 1997). This explains the highest amount of oleic acid during the season of 2009, where no rainfall was registered since March, i.e. 6 months where temperature reached 35ºC for two continuous weeks.

Gutirrez *et al*., (1999) observed a decrease in the content of oleic acid during the ripening process while Cimato *et al*., (1991) observed a constant oleic percent. The decrease appeared during the season 2009 where the oleic content started at 86.15% and finished at 68% (Graph 23); while the content plateaued during the season of 2010 between 73.15 and 76.76%. In Koroneiki cv., oleic acid decreased from green to black fruits harvested stages (Desouky *et al*., 2009). The mean values of the different fatty acids for the oils are presented in the table 21.

Table 21. Seasonal changes in fatty acid composition during the ripening process for two seasons (data are Mean ± Standard Deviation).

	Fatty acids content					
Harvest date	**C16:0 Palmitic acid**	**C16:1 Palmitoleic acid**	**C18:0 Stearic acid**	**C18:1 Oleic acid**	**C18:2 Linoleic acid**	**C18:3 Linolenic acid**
			Year 2010			
1	9.38 ± 0.24	0.67 ± 0.10	4.73 ± 0.81	76.62 ± 0.74	5.44 ± 0.82	0.29 ± 0.11
2	9.48 ± 1.75	0.36 ± 0.03	5.35 ± 0.50	76.30 ± 2.32	5.56 ± 1.31	0.50 ± 0.34
3	10.04 ± 1.24	0.65 ± 0.18	3.18 ± 0.16	75.30 ± 0.84	7.86 ±1.57	0.48 ± 0.08
4	8.86 ± 0.51	0.54 ± 0.02	3.32 ± 0.70	76.76 ± 2.20	7.52 ± 0.49	0.20 ± 0.08
5	8.43 ± 2.06	0.58 ± 0.13	3.68 ± 1.33	75.44 ± 3.51	7.98 ± 0.22	0.52 ± 0.07
6	10.84 ± 0.81	0.51 ± 0.03	3.43 ± 0.21	73.96 ± 0.96	9.39 ± 1.23	0.24 ± 0.05
7	8.69 ± 1.74	0.42 ± 0.13	3.60 ± 1.04	75.05 ± 2.86	8.41 ± 0.68	0.21 ± 0.01
8	10.13 ± 0.49	0.53 ± 0.02	3.72 ± 0.14	73.15 ± 0.22	10.48 ± 0.51	0.20 ± 0.06
9	8.66 ± 0.82	0.45 ± 0.14	4.29 ± 2.14	74.10 ± 2.82	9.40 ± 0.65	0.34 ± 0.06
10	9.36 ± 1.36	0.47 ± 0.01	4.20 ± 0.11	74.17 ± 1.06	10.28 ± 0.15	0.09 ± 0.01
			Year 2009			
1	7.19 ± 0.25	0	0.65 ± 0.03	86.15 ± 1.23	3.18 ± 0.17	0.14 ± 0.00
2	6.93 ± 0.35	0	0.75 ± 0.03	85.26 ± 4.13	4.22 ± 0.57	0.54 ± 0.14
3	6.72 ± 0.29	0	0.76 ± 0.07	82.19 ± 0.70	7.29 ± 0.26	0.47 ± 0.00
4	6.91 ± 0.54	0.04 ± 0.00	4.14 ± 0.48	75.18 ± 1.31	8.93 ± 1.06	0.14 ± 0.19
5	7.19 ± 0.88	0.31 ± 0.06	4.75 ± 0.99	69.02 ± 4.54	9.20 ± 1.71	1.23 ± 1.18
6	9.54 ± 0.55	0.34 ± 0.00	4.83 ± 0.27	68.00 ± 5.24	8.84 ± 0.61	1.57 ± 0.04
7	7.02 ± 0.63	0.38 ± 0.16	4.33 ± 0.16	68.84 ± 4.25	8.44 ± 0.28	0.72 ± 0.10
8	6.99 ± 0.03	0.53 ± 0.01	4.23 ± 0.08	68.52 ± 2.72	9.52 ± 0.69	0.76 ± 0.07

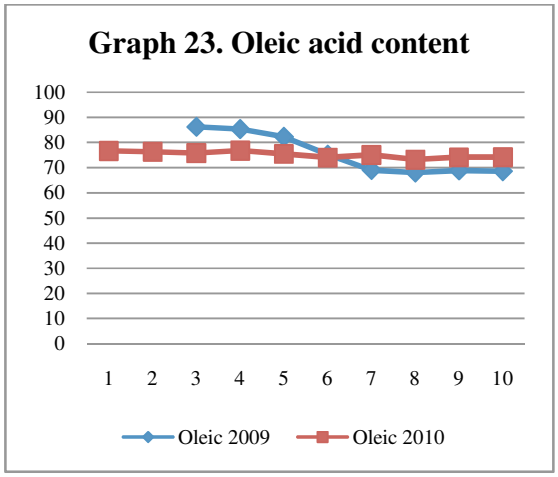
For the linoleic acid (C18:2), the content ranged from 3.18-10.22% during 2009 and 5.44-10.48% during 2010, with a mean value of 7.88. As in many olive cultivars, the level of linoleic acid increased during the ripening process (Graph 24). The content was influenced significantly by the ripening process during the two years of 2009 and 2010 and showed rapid increase during the first three collection followed by a gradual increase until the last collect when the fruits over ripe (Table 22). Linoleic acid increased respectively during 2009 and 2010 by 32.5% while the content increased by 41.2% between the second and third collect. Martinez Rivaz *et al*. (2000) observed that desaturase activity, from oleate to linoleate, was regulated by temperature during the growth of fruit, and obtained higher levels of unsaturated fatty acids for lower temperature;

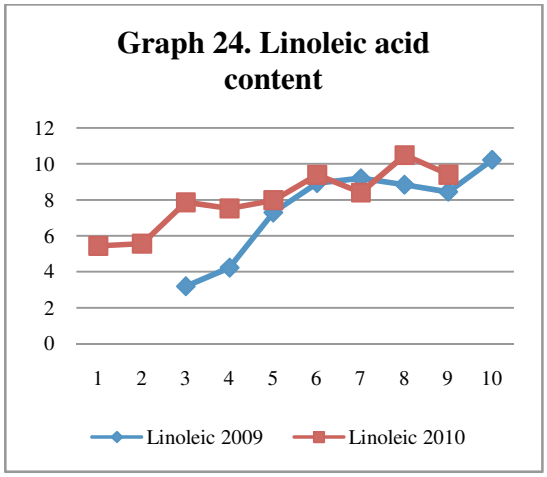
this explains the increase of C18:2 for the two crops during maturation where the temperature during October decrease a lot on 1000m altitude in Bchealehs region.

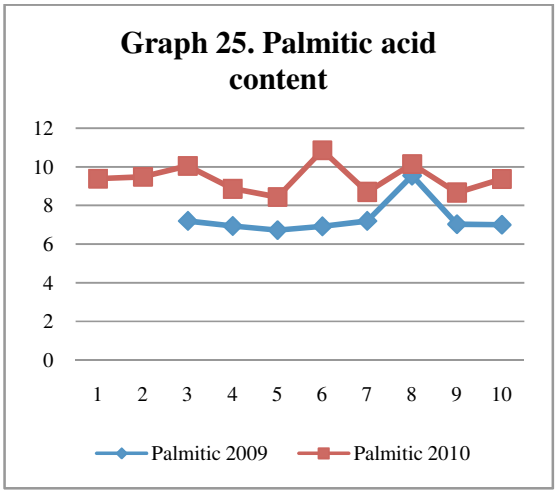
The palmitic acid (C16:0), which is the main saturated fatty acid, has a mean value of 8.35%. No differences between the two years and date of collection were observed (Table 22). The content of the palmitic acid was the lowest during 2009 and ranged between 6.72 and 7.54%, whereas the highest was found in the oil of 2010 with a range between 8.43 and 10.84% (Graph 25). These rates contradict results obtained in other cultivars, where a drop in palmitic acid content was observed during the ripening process (Vekiari *et al*., 2010; Beltran *et al*., 2004). These results are close to those of Cornibara virgin olive oil, where palmitic acid content varied between 6.99 and 10.01 (Salvador *et al*., 1998); while the level of C16:0 ranged from 11.22 in Sigoinse cv. to 19.24% in Grossane cv. (Zarrouk *et al*., 2009). Gutirrez *et al*. explained the fall of palmitic acid as a dilution effect while its quantity is constant. In Bouteillan cv., palmitic acid was too close in extracted oil from green, purple and black fruits (Desouky *et al.*, 2009).

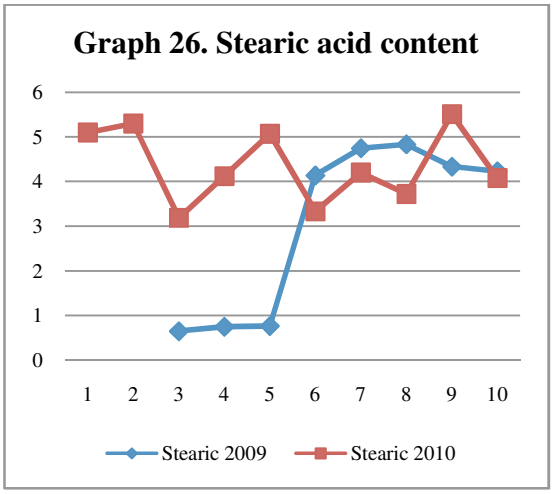
Concerning the stearic acid (C18:0), the mean values observed was 3.5% with an accumulation trend during 2009, that started during the first collect at 0.65% and rose to reach 4.83% on the sixth collect (Graph 26), this trend is similar to the one observed by Beltran *et al*. (2004), where stearic content increased during the three crop years for the Picual oil; while, during 2010 the content varied between 3.32 and 5.35% although, Salas *et al*., (1999) reported that stearic acid does not accumulate. These results differ from other olive varieties such Throumbolia cv. where the content of stearic acid didn't accumulate and ranged between 2.05 and 2.12% during the different growth stages (Vekiari *et al*., 2010).

Concerning the linolenic acid (C18:3), the highest unsaturated fatty acid, its mean value was 0.44 with respectively 0.31 and 0.57% for the year 2010 and 2009. Similarly to oleic acid, the linolenic acid may be influenced by the high temperatures in 2009 during the growth period of drupes; this could explain its higher content. No significant differences were observed (Table 22) During 2010, the linolenic content dropped similarly to the Picual virgin olive oil (Beltran *et al*., 2004).









Environmental factors such as year of harvest, season or climatic conditions, particularly temperature and rainfall during fruit growth and ripening, have great influence on the variation of some fatty acids (Leon *et al*., 2004).

Table 22. Effect of the year and collection date on the composition of different fatty acids.

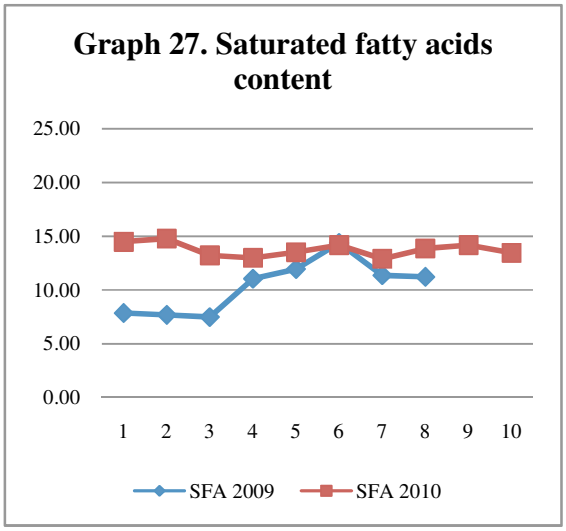
	Collection date		Year	
	F	p	F	p
Oleic	0.731	0.676	2.640	0.091
Palmitic	1.635	0.179	2.918	0.073
Linoleic	11.644	0.000***	0.086	0.918
Linolenic	1.409	0.255	1.013	0.378
Palmitic	0.941	0.515	0.334	0.719
Stearic	1.175	0.366	1.668	0.209

***** indicates significant difference from initial value at p<0.001**

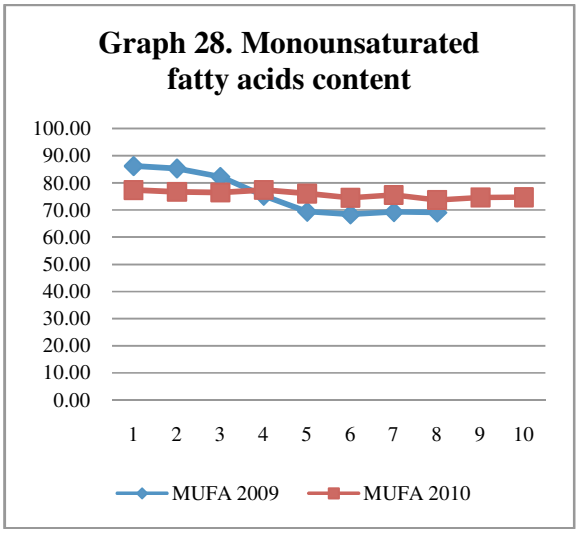
The seasonal changes in the fatty acids groups, monounsaturated, saturated, unsaturated and polyunsaturated were studied.

The monounsaturated fatty acids constitute the main group in virgin olive oil with a mean value of 75.62% and ranged between 69.05 and 86.15% (Graph 28); it shows the same seasonal variation of oleic acid during the ripening process of olives. No significant difference was observed between the two crops, the MUFAs acted similarly to oleic acid, the main constituent of MUFAs during the maturation of olive drupes.

Concerning the saturated fatty acids (SFA), an increase of the content was observed for the crop of 2010 and reached 13.44% at the end of the ripening process, while the content remained stable during 2009 after the fourth collect within the 11%. The crop yield influenced significantly the content of the SFAs with a minimum of 7.48% during 2009 and maximum of 14.78% during 2010 (Graph 27; Table 23). Since the palmitic acid constitutes the main saturated fatty acid (68.3%), the same behavior of the palmitic acid was observed on the SFA, and its total amount is also influenced by temperature.



Graph 27. Saturated fatty acids content



Graph 28. Monounsaturated fatty acids content

The polyunsaturated acid, produced by consecutive desaturation of oleic acid in olives (Vekiari *et al*., 2010), have shown contents corresponding respectively to 8.11% and 8.54% for the year 2009 and 2010. Since the linoleic acid is the main constituent of the PUFAs, the content will

have the same trend and increase during the ripening process. A significant difference is observed within the season, while the content of PUFAs increased similarly during the two seasons (Graph 29; Table 23).

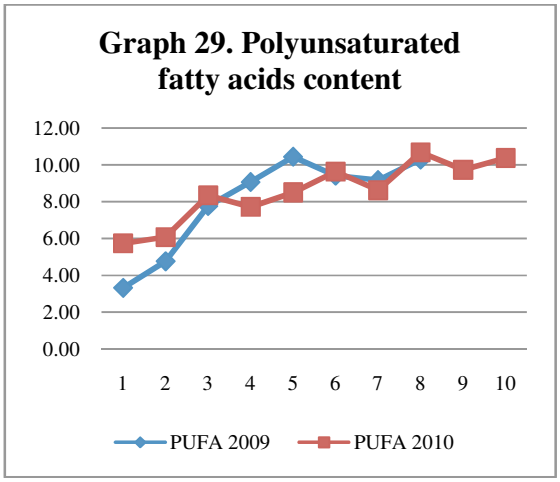


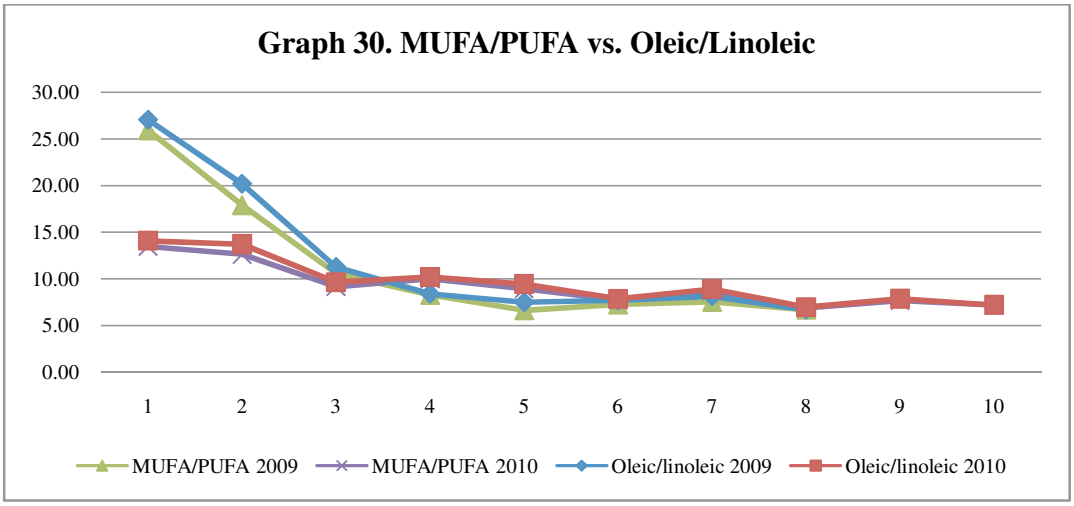
Table 23. Effect of the year and collection date on the composition of saturated, monounsaturated and polyunsaturated fatty acids.

	Year		Collection date	
	F	p	F	p
SFA	12.19	0.01*	0.602	0.766
MUFA	0.017	0.901	1.273	0.384
PUFA	0.079	0.787	7.527	0.007**

*** indicates significant difference from initial value at p<0.05; ** indicates significant difference from initial value at p<0.01**

The oxidative stability of oil is mainly influenced by MUFA/PUFA and oleic/linoleic ratios. They also have great importance due to its effects on nutritional properties (Beltran *et al*., 2004); add on that, the oleic/linoleic ratio affects the taste of virgin olive and is related to its health effects (Boskou, 1996).

Bchealehs oil had a mean value of 10.31 within a wide range that varied between 6.72 and 25.92. This difference could be attributed to high of oleic and the low linoleic acid.



The high MUFA/PUFA observed during the first three collects, was due to the high content of oleic acid and low content of the linoleic acid; then a decrease of this ratio was observed to the increase in the linoleic content till the last collect of olives (Graph 30). This result is similar to the one reported by Uceda and Hermoso who observed a decrease in MUFA/PUFA ratio due to the increase of linoleic acid and constant oleic acid value.

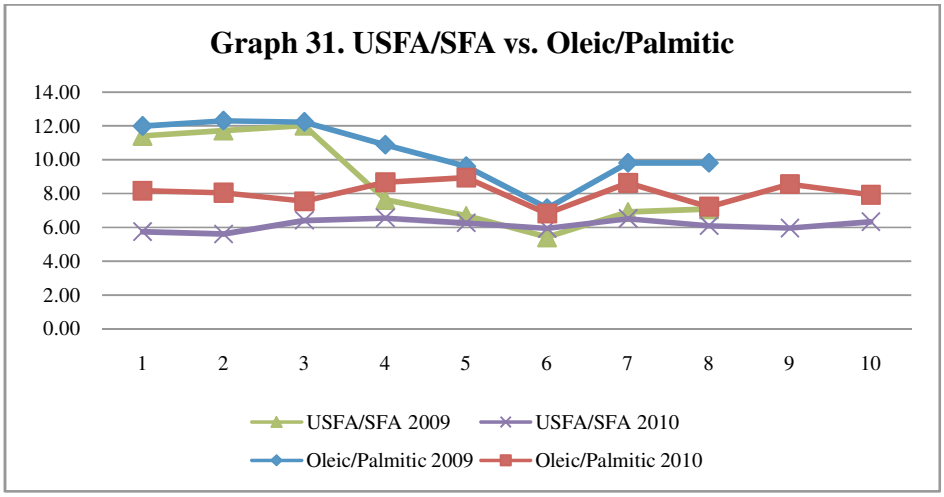
The trend of oleic/linoleic, similar to the MUFA/PUFA ratio, started in 2009 by a high ratio number (27.06) and decreased till 6.7 at the end of the ripening process. No significant difference were observed between MUFA/PUFA and oleic/linoleic ratios (Table 24). This result differs from the ones of Koroneiki and Throumboulia cv., where an increase of 9% of oleic/linoleic was observed between the different growth stages (Vekiari *et al.*, 2010). The mean value of Bchealehs ratio is 10.3 close to the ratio of Koroneiki cv. (11.3).

Table 24. Effect of the year and collection date on the MUFA/PUFA vs. Oleic/Linoleic ratio.

	Year		Collection date	
	F	p	F	p
MUFA/PUFA	0.899	0.375	2.7	0.102
Oleic/linoleic	1.201	0.309	2.902	0.087

Concerning the unsaturated/saturated (USFA/SFA) ratio, a mean value of 7.73 was observed on Bchealehs virgin olive oil. This is mainly due to the high content of oleic acid. The amount

varied between 5.6 and 6.55 during 2010, while the ratio decreased during 2009 to reach 7.1 at the end of the ripening process (Graph 31). In Arbequina cv., the USFA/SFA ratio ranged between 3.46 and 3.5, while in the Bouteillan cv., the ratio ranged between 4 and 4.2.



The date of collection didn't influence the USFA/SFA and oleic/palmitic ratios. The oleic/palmitic showed a similar behavior to USFA/SFA ratio, between 2009 and 2010, with a difference due to the accumulation of the stearic acid during the ripening process of 2009 that lead to the significant difference between years.

Table 25. Effect of the year and collection date on the USFA/SFA vs. Oleic/Palmitic.

	Year		Collection date	
	F	p	F	p
USFA/SFA	6.185	0.042*	0.642	0.737
Oleic/Palmitic	17.3	0.004**	1.281	0.38

*** indicates significant difference from initial value at p<0.05; ** indicates significant difference from initial value at p<0.01**

It should be noted that the fatty acid composition meets the limits set for extra virgin olive oils in the EC regulation and IOOC standards.

4.4.3. Acid index changes during fruit ripening.

During fruit maturation, and as ripening progressed, a significant increase of the free acidity was observed. The acid index acted similarly and increased during fruit ripening (Table 26). At a maturity index close to 1, the acidity index increased from 0.11 to 0.44 at a maturity index close to 6. The same behavior was observed on selected oleasters (Baccouri *et al.*, 2007), and other varieties such as Picual (Gutierrez *et al.*, 1999) and Arbequina (Garcia *et al*., 1996).

Table 26. Effect of the year and collection date on acid index.

	Year		Collection date	
	F	p	F	p
Acid Index	0.02	0.89	112.98	0.000***

***** indicates significant difference from initial value at p<0.001**

During all the ripening process, the acid index was lower than the limit established by the IOOC that must be lower than 0.8 in order to be classified as extra virgin oil. A significant difference was observed during the ripening process. For 2009, the free acidity varied between 0.12-0.41, while during 2010, it varied between 0.1-0.55 at different harvesting stages. No significant difference within the crop year.

At later stage, the acidity percentage increased especially in black stage with the highest acidity percentage, respectively 0.41 and 0.55 in 2009 and 2010; when drupes over ripe, an increase in lipolytic activity took place. The olives become more sensitive to pathogenic infections causing an increase in the free acidity. This explains the increase in acid index after an unchanged value between the sixth and the eighth collect (0.36-0.39).

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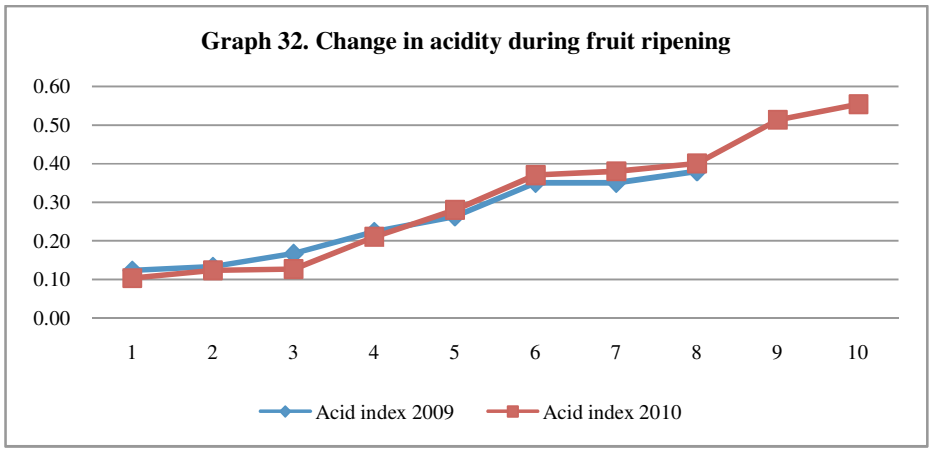


Acidity, which is the percentage of fatty acids released by hydrolysis of triacylglycerols chains, is gradually changing with lipid accumulation during the ripening of olives. The increase in the acidity was also observed during ripening on other studied varieties such as Chemlali and Zalmati Zarraris. Ben Salah *et al.* (1986) noted that acid index of olive oil harvested in mid-March increased in the same trend of the lipase action during the ripening.

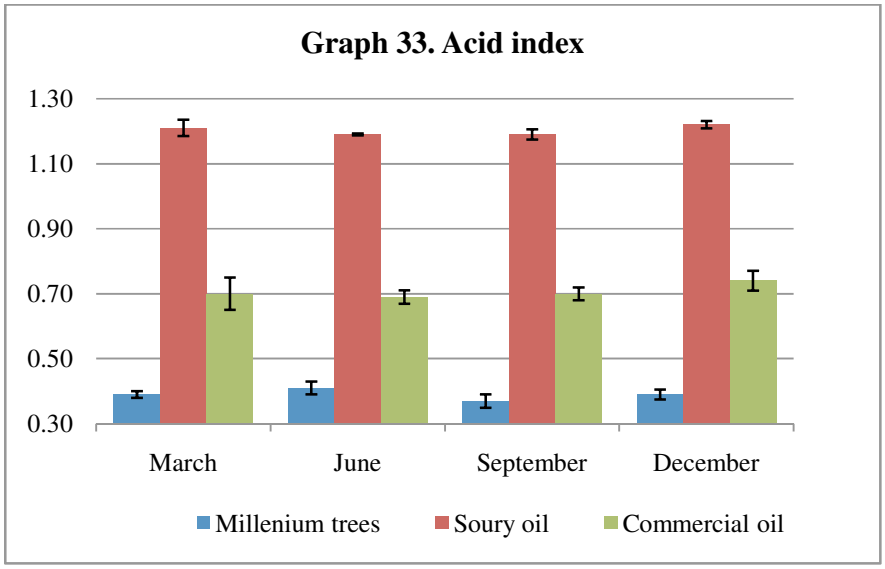
4.5. Effect of conservation on olive oil parameters.

Starting March 2010 and for one year, the influence of conservation on the olive oil indices of Bchealehs and Soury oils was observed.

4.5.1. Acid Index.

The initial values of the three oils, which ranged between 0.39 and 1.21, were within the limits allowed by the EC regulation. For the acid index, Bchealehs oil had the lowest mean value with 0.39, followed by the commercial oil 0.71 and the Soury oil 1.2 (Graph 33).

The three oil acted similarly and didn't show any variation all around the year (Table 27). The relatively low free acidity observed in Bchealehs oil, is due to healthy fruits and to their rapid processing, while the higher acidity of Soury oil could be due to the non pulverization of the orchards that led to deterioration in the drupes caused by *Dacus olea*.



The results showed that there was no significant increase in oil percentage as conservation period progressed. A highly significant difference was observed between different types of oil (Table 27).

Table 27. Effect of testing date and cultivar on acid index.

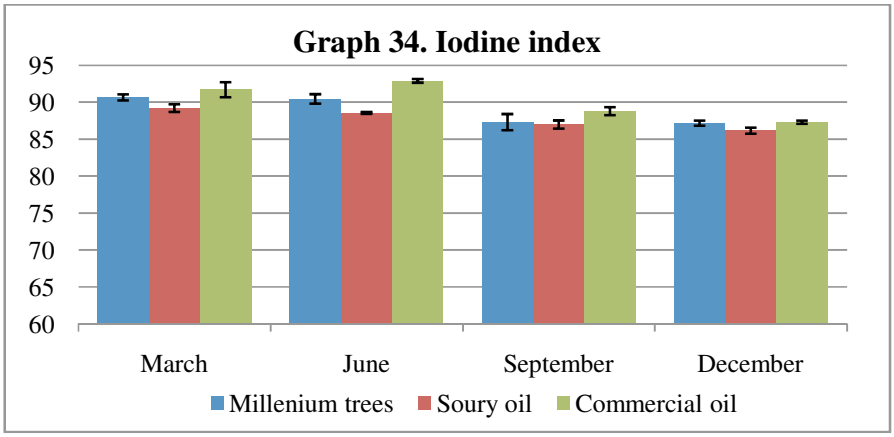
	Month		Cultivar	
	F	p	F	p
Acid Index	2.588	0.148	4548.281	0.000***

***** indicates significant difference from initial value at p<0.001**

4.5.2. Iodine index.

The higher the iodine index, the more unsaturated is the oil. It indicates the high level of polyunsaturated fatty acids. Lipids with unsaturated fatty acids (containing one or more double bonds) are easily assimilated and broken down to produce calorific energy than saturated fatty acids. However, when the iodine index becomes too high, the stability of the oil reduces because it'll easily undergo oxidation.

This high iodine index in Bchealehs oil is probably due to its high level of linolenic acid (10.28%). The iodine index of the oils ranged from 86.2 to 92.94g/100 g of oil (Graph 34).



The iodine index was highly influenced by the testing date. All the oil acted similarly and decreased to reach the minimum value one year later. Soury oil had the lowest value (86.2). No difference was observed, during December test, between Bchealehs and commercial oil with respectively 87.21 and 87.34 (Graph 34). The cultivar influenced significantly the iodine index (Table 28).

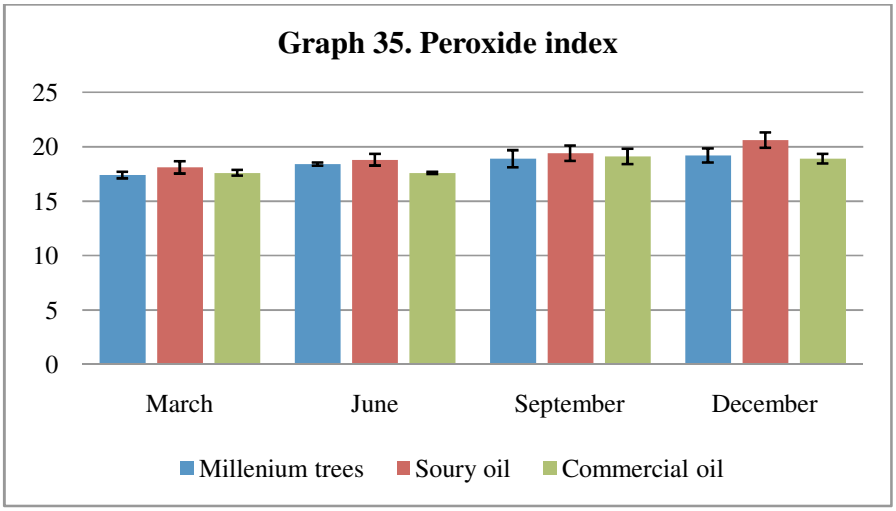
Table 28. Effect of testing date and cultivar on iodine index.

	Month		Cultivar	
	F	p	F	p
Iodine Index	237.739	0.000***	21.161	0.007**

**** indicates significant difference from initial value at p<0.01; *** indicates significant difference from initial value at p<0.01**

4.5.3. Peroxide index.

The peroxide index, which is an indicator of the hydro peroxides amount present in oil, ranged in the three oils from 17.4 to 20.6. Nine months after the extraction of Soury and Bchealehs oils, the peroxide values were lower than 20meq/kg of oil, the maximum level set for cold pressed and virgin oils (Codex Alimentarius, 1999) (Graph 35). These high values could be attributed to the effect of storage. The highest value of Bchealehs oil observed one year after its extraction (19.2), during December 2010, was still below the limit (20 meq O_2/Kg oil).



The peroxide index increases as the fruit matures. For Bchealeh cv., the high peroxide value during the first test (17.4) is mainly due to the black drupes from which oil was extracted. The peroxide value of Bchealeh increased significantly from 17.4 on March 2010 to 19.2 on December 2010 (Graph 35; Table 29).

The testing date and cultivar influenced significantly the peroxide index of the three oils which increased in the three oils from the first till the fourth test. One year after its conservation, oil that had the lowest peroxide index was the commercial oil (18.9), followed by Bchealehs oil (19.2) and Soury oil (20.6).

The peroxide value depends on the mode of oil extraction, the type of fatty acids present in the oil, the preservation mode and the quantity of oxygen consumed. Oil that contains high polyunsaturated fatty acids could easily undergo oxidation, raising peroxide values in these oils.

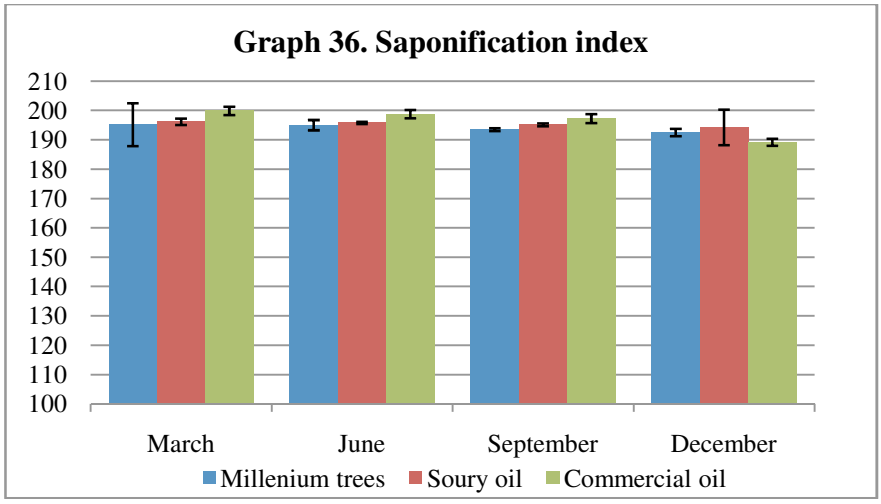
Table 29 . Effect of testing date and cultivar on peroxide index.

	Month		Cultivar	
	F	p	F	p
Peroxide Index	24.188	0.001*	7.244	0.047*

*** indicates significant difference from initial value at p<0.05; ** indicates significant difference from initial value at p<0.01**

4.5.4. Saponification index.

Concerning the saponification index of the samples, the values exceeded the range fixed by the EC which is 182-193mg KHO/g oil. The commercial oil had the highest mean value (196.2), followed by Soury oil with 195.3 and Bchealehs oil with 194.1. The values ranged between 192.5 and 199.9 (Graph 36).



The testing date influenced significantly the saponification index (Table 30). The first difference was observed three months after the extraction of oils. Twelve months later, a decrease in the three oils was observed; Bchealehs index decreased from 195.2 to 192.5, while the commercial oil showed the biggest decrease and passed from 199.9 to 189.2. Soury oil index decreased from 196.2 to 194.3. Concerning the type of oil, it didn't show any significant differences and all the oils acted similarly within a maximum difference of 5.4% for the commercial oil, 1.3% for the Bchealehs oil and less than 1% for the Soury oil (Table 30).

Table 30. Effect of testing date and cultivar on saponification index.

	Month		Cultivar	
	F	p	F	p
Saponification Index	8.817	0.013*	1.345	0.357

*** indicates significant difference from initial value at p<0.05**

5. Conclusion.

The conservation of Lebanese genetic resources constituted the main purpose of this study and consisted on the *in vivo* and *in vitro* introduction of Bchealehs cuttings. Being the eldest olive trees in Lebanon, it was a challenge to produce rooted plants especially that the juveniles' branches are not in good shape and didn't show a good vegetative growth.

***In vitro*.**

The *in vitro* initiation phase quickly encountered contamination problems, fungal and bacterial, causing the loss of the majority of the plant material. This issue was resolved by applying a pretreatment in the nursery using two fungicides propamocarb-HCl and metalaxyl+mancozeb, followed by a treatment *in vitro* and composed of a mixture of two fungicides carbendazim and fosethyl-aluminium, two bactericides NaOCl (15%) and Chloroxylenol (2.5%), and one antibiotic the oxytetracycline (1mg/l); this treatment resulted in a decrease of the contamination amount (43.3%) and a higher number of proliferated explants. This result is lower than the one obtained by Sakunasingh *et al*. (2004), (80%) when only one fungicide (Benomyl 2%) and one single detergent (25% NaOCl) were used. This difference could be due to the absence of the fungicide application on olive groves in order to lower production costs.

Once the appropriate treatment applied, the work then focused on the adaptation, multiplication and rooting of the plant material. During the culture initiation and independently of the media and the concentration of BAP used, the highest percentage of broken buds was observed on MS media with 40.27% of proliferated buds followed by WPM (37.8%) and OM (35.4%). The autumn season was the best for the initiation phase and gave the highest amount of proliferated buds with 47% and newly developed explants (1.35). Bougdal *et al.* (2007) also noted that the period of active growth of olive trees was situated in the fall season.

Concerning the multiplication phase and during the three tested seasons of 2010, the first and second subcultures gave the same and highest proliferated rates with 0.93 new explants, while this rate decreased to 0.71 during the third subculture. MS media was more beneficial than the two media OM and WPM for the multiplication rate of Bchealehs explants with an average of

1.08 new explants on MS added with 3mg/l BAP while the lowest rate (0.64) was observed on WPM added with 4mg/l BAP. OM (olive medium) and WPM (woody plant medium) were respectively more beneficial in Chalkidikis cv. (Grigoriadou *et al*., 2002), Maurino cv. (Bartolini *et al*., 1989). These results indicate that genetic factors are decisive in the choice of culture medium (Brhadda *et al*., 2003).

Season also had a significant influence on the multiplication rate where autumn season gave the highest amounts of new explants during 2009 and 2010 with respectively 0.94 and 0.8. Results are similar to the initiation phase where the highest percentage of broken buds was observed during the autumn season.

The longest explants (1.78cm) and the highest number of newly developed leaves (3.58) were observed during the first subculture on the MS media added with 3mg/l BAP, while the smallest explant (1.43cm) and the lower number of leaves (2.6) were respectively registered on WPM3 and WPM1.

Concerning the rooting, NAA gave the highest amount of rooted explants (22% of the transferred explants) when added to MS medium at a rate of 2mg/l. NAA was the most favorable auxin for the explant rooting while IAA didn't exceed 6.25%.

Bchealehs explants have shown difficulty in proliferating the callus that appeared during autumn season even when proper concentration of kinetin and NAA were applied to the medium. All callus, when transferred to fresh media, were lost by oxidation before developing any seedlings.

When kept on the explant, this callus facilitated the rooting of 23% of the plant material without passing by any subculture. This could be an alternative technique of the *in vivo* culture which needs lot of cares and must ensure an optimal temperature and humidity.

***In vivo*.**

The *in vivo* rooting ability of Bchealehs cuttings was affected by cutting size, collection season and medium used. The collected material with a diameter < 0.5cm didn't develop well. Only

cuttings which has a diameter > 0.5cm proliferated. The highest (88%) and lowest (40%) percentage of emerged buds were recorded on Bchealehs cv. during 2010, while this rate didn't exceed 66.7% during 2009.

The Soury cv. showed its superiority during autumn season and this concerning the length (5.73%), the number of leaves (14.6%), the percentage of callused (46.7%) and rooted (30%) explants. Another media should be tested, especially that roots didn't develop well in the mix of peat moss and potting soil. The mean value of rooted Bchealehs cuttings during 2010 was 10.67%, while this amount didn't exceed 4% during 2009. Rooted cuttings appeared only two months after the introduction of plant material *in vivo*. The rooting ability between years has been ascribed to the alternate bearing trend shown by mature stock plants which might be related to the endogenous carbohydrate status (Ozkaya and Celik, 1999).

For economical usage, focus should be placed in deciding the appropriate cutting size and rooting media.

The use of NAA wasn't efficient enough and gave at the end of the experiments only 7% of rooted explants. These results contradicts Fernandez *et al*. (2002), who showed that the use of 0.2% NAA powder was more effective on difficult to root "Galega Vulgar" than treatment with 5000ppm liquid IBA. Treatment of other olive cultivars showed that NAA wasn't sufficient when applied alone in quick dip; its use mixed with IBA could be more beneficial for the increase of root formation in olive cuttings.

Olive parameters.

The Bchealehs cv. gave olives with good characteristics for the production of oil and olives tables especially by the end of November 2010, where the highest oil content reached 28.96% and the recorded flesh/pit ratio and weight were respectively 6.63:1 and 4.31g. During maturation of the fruit, and when olives turned from green to purple color, weight, fruit width, flesh/pit ratio, shape index and oil percentage increased significantly and then a slight decrease was observed when olives turned totally into black and overripe. The parameters difference between 2009 and 2010 is mainly due to the heavy yield of 2009 that influenced the weight, size

and flesh/pit ratio. The percentage of oil remained close and passed from 28.9 to 26.5%. These valuable parameters, when compared to other Lebanese olive cultivars, could make Bchealehs cv. a highly recommended in the establishment of new orchards.

Olive oil composition.

The fatty acids composition of the analyzed oil fell within the ranges established for ‘‘extra virgin olive oil’’ category. The collect period, and thus the ripening stage, didn’t affect significantly the oleic, palmitic, stearic, and linolenic acids; while only linoleic acid was influenced by the date of collect. Due to the increase of linoleic acid during maturation, the delay of olive harvest allows us to get a better oil quality. The non significant difference could be due to the close interval, which was one week, between one collect and another.

The MUFA/PUFA and oleic/linoleic ratios of Bchealehs oil showed a highly significant relationship and decreased with ripeness. This result is similar to the one observed on Picual cv. (Beltran *et al*., 2004).

The observed results of olive parameters and olive oil composition could be used for the determination of the optimal harvesting date, set immediately prior to the color change from purple to black and when oil content and flesh/pit ratio reached their maximum levels.

Olive oil conservation.

Bchealehs samples showed very low values for the regulated physicochemical parameters one year after its conservation with maximum recorded value of free acid (0.41); peroxide value (19.1 m equiv. O_2/kg) and iodine value 87.2; all of them fell within the ranges established for ‘‘extra virgin olive oil’’ category, as required by Codex Alimentarious, (2003). Add on that, the quality of Bchealehs oil met the IOOC standards for exportation of extra virgin olive oil.

Changes that occurred in the Bchealehs oil during the conservation could influence later the shelf life potential and sensory characteristics if conserved for more than one year. These parameters had a significant effect on the stability of the oil unless the values stay below the norms set by Lebanese Standards Institution.

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