IN VITRO ORGANOGENESIS AND EMBRYOGENESIS OF PISTACHIO, *PISTACIA VERA* L.

by

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Declaration

I hereby declare that this thesis has been composed entirely by myself and all that work herein described to be my own, except where otherwise acknowledged.

Ahmet Onay

Edinburgh 1996

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Publications

Onay A, Jeffree CE, and Yeoman MM 1995 Somatic embryogenesis in cultured immature kernels of Pistachio, *Pistacia vera* L. Plant Cell Rep 15: 192-195.

Onay A, Jeffree CE and Yeoman MM 1995 Plant regeneration from encapsulated embryoids and an embryogenic mass of pistachio. Plant Cell Rep (*in press*).

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List of abbreviations and symbols

Name	Synonym
Auxin	
2,4,-Dichlorophenoxyacetic Acid	2,4-D
2,4,5,-Trichlorophenoxyacetic Acid	2,4,5,-T
α-Naphthaleneacetic Acid	NAA
β-Naphthoxyacetic Acid	NOA
Indole-3-acetic Acid	IAA
Indole-3-butyric Acid	IBA
Indole-3-propionic Acid	IPA
Picloram	
p-Chlorophenoxyacetic Acid	CPA
Cytokinin	
Adenine	
6-Dimetylaminopurine	2iP
6-Benzylaminopurine	BAP
Diphenylurea	
Kinetin	K
1-Phenyl-3-(1,2,3-thiadiazol-5-yl) Urea	Thidiazuron (TDZ)
Zeatin(Zeatin-Mixed isomers)	Zea
Other plant growth regulator	
Abscisic Acid (+,–) Cis-trans Isomer	ABA
3,6-Dichloro-o-anisic Acid	Dicamba
Colchicine	
Gibberellic Acid	GA_3
Activated charcoal	ac
Anderson's Rhodendron Basal Salt Mixture	A
and other	et al.
Approximately	c.
Boron	B
Calcium	Ca
Centimetre	cm
Citric acid	CA
Chi-square test	χ^2
Day	d
Degrees of freedom	df
Degree Celsius (centigrade)	°C
Editor (s)	Ed(s)

Embryogenic Mass(es)	EMS(es)
Embryonal suspensor mass	ESM
For example	e.g.
Gamborgh's B-5	G-5
Hour (s)	h
Hydrogen peroxide	H_2O_2
Hydrochloric acid	HCl
Kinetin	K
Kilogram	Kg
l-Ascorbic Acid	AA
Micrometer	µm
Micromolar	µM
Millilitre	ml
Millimeter	mm
Milligramme per litre	mgl^{-1}
Millimolar	mM
Month	mon
Murashige and Skoog medium	MS
Multiple Shoot Formation	MSF
Nanometer	nm
Negative logarithm of the hydrogen ion concentration	pH
Number	No
Not determined	N.D.
Not significant	N.S.
Plant growth regulator(s)	PGR(s)
Page/pages	p/pp
Percentage	%
Probability	P
Polyvinylyprolidone	PVP
Rotation per second	rps
Rotation per minute	rpm
PSI	$1b/inch^2$
Schenk and Hildebrand medium	SH
Second	s
Somatic embryo(s)	SE(s)
Stored clones	SC
Standard error ±	SE ±
That is	i.e.

Volume per volume	v/v
Volume per weight	v/w
Woody plant medium	WPM
Watt	W
Year	yr

Glossary

Adventitious meristem: A meristem arising from disorganised cells.

Adventitious: Tissues or organs developing in an abnormal position, i.e. buds produced at places other than from leaf axil and roots growing from stem or leaves.

Autonomous: Self-supporting; independent.

Auxin: Plant hormone traditionally considered to promote cell enlargement and elongation e.g. IAA (natural) and 2,4-D (synthetic).

Axillary buds: Those formed in the axil of the angle between a leaf and the stem.

Axillary meristem: Meristem giving rise to axillary buds.

Bud: An undeveloped shoot covered with protecting scales, consists of a very short shoot axis and primordia of leaves or floral parts.

Callus: Actively growing relatively undifferentiated tissue, devoid of macroscopic organised structures, normally produced in higher plants in response to wounding or infection, but often formed *in vitro* during the artificial culture of plant tissue.

Cell-line: A propagated cell culture having relatively stable characteristics, in extremely variable cultures, each individual flask may constitute a distinct cell line.

Chelating agent: An organic compound that is capable of complexing with a metal on to form a chemical structure in which a central polyvalent metal ion is combined with a ring of organic compounds or radicals.

Clonal propagation: Asexual propagation from the same original cells or organism (ortet) of many new plants (ramets) all with the same genome or genetic material.

Clusters: Group of things of the same kind growing naturally together; bunch; pistachio nuts grow in a cluster.

Cytokinin: Plant hormone traditionally considered to promote cell division and differentiation e.g. Zeatin (natural) and 6BA (synthetic).

Dedifferentiation: The losing of characteristics of specialised cells and regression to a more simple state. Often has the meaning of reprogramming of cells to a state in which they are capable of adventitious organ or embryo formation.

Differentiation: The general process by which unspecialized cells change in structure to perform a specialized function in the tissues or organs of an organism.

Elite organism: An individual plant (or organism in general) that possesses desirable characteristics that are not shared by the majority of that species or variety.

Embling: A term used to describe germinated somatic embryos and is intended to differentiate from seedlings germinated from analogous zygotic embryos.

Embryogenesis: Process of embryo initiation and development to an embling from a cell culture.

Embryogenic: Relating to the nature of an embryo.

Embryogenic mass: Actively growing clusters of green meristematic tissues, formed *in vitro* during the artificial culture of plant tissue.

Embryoid: Embryo-like structure, which may grow into a plantlet.

Endocarp: The shell surrounding the kernel. Inner most layer of pericarp.

Endosperm: Storage tissues of the seed. Food reserve for the embryo.

Explant: That part of a plant that is excised for use in the initiation of a cell or tissue culture.

Genotype: Genetic constitution of an individual carried by the DNA of the cell nucleus.

Germination: The period between excision of the embryo from EMS and the stage at which the shoot emerges and adventitious roots are formed (normal development).

Heterotrophic: Of an organism which requires a supply of a carbon compound as a source of energy and for growth such organism usually cannot fix carbon dioxide in the light.

Heterozygous: An organism or cell showing two different alleles at a corresponding location on homologous chromosomes.

In vitro: Lit. 'in glass': culture in vitro = culture (typically) in glass vessels providing a sterile artificial environment.

In vivo: In the natural conditions in which organism reside.

Kernel: Endosperm plus testa and embryo.

Meristem: A group of small, actively-dividing cells the derivatives of which differentiate into the tissues of the plant body. Root and shoot apical meristem are highly organised structures. The latter give rise to precise patters of buds and leaves.

Meristematic: Having meristem like characteristics.

Meristemoid: An active locus of growth; a nodule of undifferentiated tissue from which new cells and/or adventitious structures arise.

Micropropagation: Rapid vegetative propagation of a plant via small pieces of tissue, and usually beyond that obtained in nature.

Organogenesis: Initiation of an organ, or the production of a plantlet *in vitro* through the sequential, usually non-synchronised initiation of root and shoot structures connected by vascular tissue.

Plantlet: A tiny plant with a distinct root and shoot system formed via tissue culture either by embryogenesis or organogenesis.

Regeneration: Development of cultured tissue, cells or protoplast towards the whole plant. It can refer to wall resynthesis or colony formation in protoplasts, an organ or whole plantlet formation in cells or tissue.

Seed: Fruit with the mesocarp removed, consisting of endocarp and kernel.

Somaclonal variation: Genetic, epigenetic and phenotypic variation among somatic cells and micropropagules, recorded as a result of genetic differences pre-existing in somatic cells or induced by the environment within the cultured medium.

Somatic embryo: An embryo-like structure originating from a somatic (non-germ) cell.

Somatic hybrid: A hybrid resulting from the fusion or non-germ cells.

Tigellum: Portion of embryo containing root and shoot meristems.

Tissue culture: A general term used to describe the development of tissues in culture under sterile conditions.

Totipotency: The ability of a somatic cell *in vitro* to regenerate a whole organism either via organogenesis or embryogenesis.

Undifferentiated (tissue cultures): Showing no morphological cellular specialisation e.g. vascular elements, cell alignments, root or shoot formation etc. the term cannot be used in an absolute sense because biochemical specialisation may easily go unnoticed.

Variant/mutant: An organism showing a stable phenotypic derivation from the wild type. Such an organism can only be classed as a true mutant if an equivalent change in the primary structure of the genome can be demonstrated.

Vegetative (cell): All cells other than germ cells.

Vegetative propagation: Somatic non-sexual propagation of plant parts without fertilisation.

Vitrification: A physiological disorder associated with specific changes in the appearance of induced organ *in vitro*, where leaves become translucent, appearing glassy or water-logged.

Zygotic embryo: An embryo arising from a fertilised ovule as in the normal life cycle of the plant.

Abstract

Methods were developed for organogenesis and somatic embryogenesis of pistachio, *Pistacia vera* L. cv 'Antep', using tissues from seedlings, mature trees, immature fruits, zygotic embryos and juvenile leaf explants. An effective surface sterilisation method for the production of sterile explants from *P. vera* mature seeds or immature fruits, seedlings, and mature meristem tips was achieved. In the case of explants from adult pistachio materials, decontamination was best achieved when explants were obtained from actively-growing meristem tip cultures of 50-year-old *P. vera* L. A method was described for the establishment of embryogenic cell suspension cultures. The factors controlling the initiation, maturation, germination, embling development, and acclimatisation of emblings derived from immature fruit explants were investigated. The cytokinin BAP was found to be essential for the induction of EMS from immature fruits cultured on a liquid MS medium. The best growth of EMS in terms of fresh and dry weight production was obtained on sucrose or glucose within the concentration range 4-10% w/v. Somatic embryos were found to mature more rapidly in liquid medium. An original method of logistic analysis was developed for interpretation of the effects of multiple treatments and their interactions on the probabilities of embryo germination and embling development. The abscisic acid and benzylaminopurine concentrations, the durations of the embryo maturation treatments and of the culture periods for germination and embling development inluenced the most significant effects on embryo germination and embling development. The overall probability of germination of a mature somatic embryo was found to be 0.38 and 0.46 when ABA and BAP respectively were used for SE maturation. Under the germination conditions, the overall probability of an embling developing from a germinated embryo was found to be 0.18 and 0.36 for SE maturation using the growth regulators ABA and BAP. However, the probabilities of SE germination and embling were highest when PGR-free media were used for SE maturation. A practical procedure was developed for short-term storage of encapsulated EMS and somatic embryos. EMS fragments, induced in culture from immature fruits of pistachio, were encapsulated in calcium alginate beads. Somatic embryos were similarly encapsulated individually in calcium alginate beads to produce synthetic seeds. The conversion frequency of synthetic seeds to seedling plants was 14% after storage for 60 days at 4°C. The somatic embryogenic potential of axenic-juvenile, regenerated greenhouse-house grown (one-year-old) and regenerated mature leaf explant of pistachio was investigated on MS medium supplemented with different cytokinins and auxins. TDZ was found to be the most effective growth substance for the induction of rapidly growing callus. Embryogenic callus was induced from axenic juvenile leaf explants. Induction of embryogenic competency was dependent on the presence of the cytokinin BAP. Methods are described for somatic embryogenesis and subsequent plant regeneration from mature zygotic embryos with an intervening callus phase. A concentration of 1-4 mg l^{-1} 2,4-D was found to be a satisfactory growth regulator for producing viable callus and this may be used routinely in callus

induction, but BAP was the most effective growth regulator to maintain callus. An embryogenic type of callus was formed when zygotic embryos were cultured on medium containing 2,4-D or 2,4-D plus BAP and regularly subcultured on BAP containing medium. Only MS medium supplemented with BAP led to somatic embryogenesis, and other media and sugars did not improve callus texture. Chromosome counts in zygotic embryos were revealed as a complement of $2n = 2x = 30$. The diploid chromosome number in all regenerated plantlets clearly indicate that there was no numerical variation in the complements of regenerated plantlets. The results are discussed in relation to a strategy for cloning elite mature pistachio trees. It is encouraging that several protocols for pistachio regeneration through somatic embryogenesis have been established. This provides a sound basis for the adaptation of more sophisticated culture methods for elite mature trees of pistachio. However, the exploitation of somatic embryogenesis for cloning requires developing protocols to regenerate and/or rejuvenate mature elite-trees under *in vitro* conditions. It will not be possible to realise the potential without further extensive research on somatic embryogenesis.

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CHAPTER ONE

INTRODUCTION

CHAPTER ONE : INTRODUCTION

1.1 : GENERAL INTRODUCTION

Pistachio (*Pistacia vera* L.) is an important nut crop widely cultivated in the semiarid region (s) of Middle Eastern and Mediterranean countries, and the United States of America. Pistachio nut tree requires a very special climatic condition; cold in winter and warm in summer. The area that is suitable for pistachio nut production is therefore limited in Turkey and elsewhere in the world (Ayfer 1990). According to Ayfer (1963 and 1990), the pistachio growing areas which have an average temperature around (7.0-7.4°C) during winter or the average chilling requirements of this species around 800-1000 h together with average summer (June, July and August) temperature should be above 30°C for 98-110 days in a year which is suitable for pistachio nut trees (Ayfer 1963). *Pistacia vera* L. is an Irano-Turanian species, the main range of which covers the middle Asian republics of Uzbekistan, Tadzhikistan, Kirgiziya and southern most parts of Turkmenia and Kazakhstan. In the north, *P. vera* extend to about latitude 43° N. to the Karatau, Kirgizskiy and Talasskiy Alatau mountain ranges, while in the south and south-western Afghanistan, in the Paropamisus Mts. in the Herat province it reaches a latitude of 35° N. It grows most abundantly and on the most extensive areas in Tadzhikistan, where it occupies about 115.000 ha; in the whole of central Asia natural thickets cover about 300.00 ha (Browicz 1988). In Turkey, the predominant commercial cultivars are "Antep" (pistillate and staminate, Fig. 1 and Fig. 2, respectively), "Siirt", "Bilgen", "Kellegouchi", "Mumtaz", "Ohadi", "Sedifi", and "Vahidi". "Antep" is the main variety in Turkey. Some Iranian cultivars such as "Ohadi", "Kellegouchi and "Mumtaz" are propagated in South-east Turkey but are still in an experimental phase and due to their bigger fruit size, some of these Iranian cultivars may increase in importance. In Turkey, the pistachio is cultivated under non-irrigated land conditions. The pistachio nut is an important crop in Turkey for many reasons (Kuru 1990). Firstly, Turkey is one of the centres of genetic diversity of pistachio, which is very well adapted to the ecological conditions of the country. Secondly, the pistachio nut is quite nutrient rich. Thus there is an increasing demand for the nuts, and trade in them is increasing. Finally it is an important export product of Turkey. There are about 66 million wild pistachio trees that are suitable for the grafting of cultivated pistachio trees in Turkey (Kuru 1990). Two thirds of them are growing in forest and the remainder in barren areas. It has been observed that in many forest the wild pistachio grow more densely than in the newly-established pistachio orchards. Moreover, it has been estimated that 52 million commercial pistachio trees could be grown in the open patches in the wild pistachio areas (Kurt 1990). As has been perceived, it is a significant shortcoming that exploiting this potential to contribute to national income has not been exploited as yet.

Figure 1 : *50-year-old pistachio tree (P. vera cv. antep, pistillate) growing at the Ceylanpinari state production farm, in the Urfa province of South-east Turkey.*

Figure 2 : *50-year-old pistachio tree (P. vera cv. antep, staminate) growing at the Ceylanpinari state production farm, in the Urfa province of South-east Turkey.*

Today, the major limitation facing the widespread expansion of commercial pistachio plantations is the shortage of regenerated superior plant material and this has been made more acute by the difficulties which are experienced in propagating this species using the traditional method of grafting. In addition, grafting is slow and expensive, thereby limiting the number of propagated plants which can be produced. Moreover, the South-east Anatolia Project (GAP), which is one of the biggest irrigation schemes in the world, creates an irrigated land opportunity for pistachio nut production. Within a decade or two the GAP in the sun-based Tigris and Euphrates river basin is planned to reach a target irrigation area of 4.2 million acres, available to all forms of agricultural production including cultivation of pistachios (Pope 1994). Hence, there is a perceived need for new commercial cultivars which may be adaptable to irrigated land because, as pointed out above, pistachio nut tree requires very special climatic conditions. The pistachio industry may thus face some new challenges in developing this potential. In the context of the pistachio industry, one of the approaches to solving these problems of supply of desirable plant material is to use an intensive clonal propagation of pistachio trees through plant tissue, organ and cell culture techniques from which the pistachio could benefit just as some species (*Carica candamarcensis, Phoenix dactilifera* and *Simmondsia chinensis* - Jordan et al. 1983; Reynolds 1982 and Chaturvedi and Sharma) have already done.

As will be pointed out in the following subsection, in the intensive clonal propagation of pistachio rootstocks and cultivars, the possibility of the application of methods has been extensively investigated through organogenesis. Unfortunately, despite more than a decade of research, no practical process has been achieved for the vegetative propagation of mature pistachio species via tissue culture techniques.

1.2 : STATEMENT OF AIM AND OBJECTIVES

The **aim** of this study is the development of an effective and reproducible method for vegetative propagation of mature *Pistacia* species which may be used routinely through tissue culture techniques.

In the context of the pistachio industry, the original **objectives** of the present study are, therefore, as follows:

a) the prime objective is to achieve an alternative methods which may be used to extend the existing micropropagation strategies available to seedlings and mature fruit-bearing pistachio trees old enough to have expressed their superior characteristics.

b) the second objective is to achieve an alternative method of pistachio improvement by means of embryogenesis of preferred genotypes.

1.3 : REVIEW OF THE LITERATURE

1.3.1 : Origin and History

The origin of the pistachio was not known up to the beginning of the present century. The Queen of Sheba during her visit to Assyria commandeered the limited crop of nuts for her exclusive use and that of her guests (Whitehouse 1957). According to Dioskurides, the latin word pistachio is derived from "pissa" = resin and "aklomai" = to heal. That is to say, it is a plant with wholesome resin. Another explanation given by Davatchi (1958) sees its origin in the Persian word "peste". However, it is generally acknowledged (Moldenke and Alma 1952) that the nuts of Jacob were pistachio nuts, called Gatoum = batam by the Arabs. Currently, the pistachio nuts are called "Antep fistigi" by the Turks because they are mainly planted in Antep province in the South-east Turkey.

Botanists such as Linnaeus, Candolle, Boissier and Engler did not know of the existence of pistachio savannahs. Syria and Mesopotamia were considered as the natural habitat of pistachio (Bailey 1947). According to Vavilov (1951) the origin of pistachio are:
(1) central Asia, including north-east India, Afghanistan, Tajikistan and Uzbekistan,
(2) the near-east which covers Asia Minor, Caucasus, Iran and the mountain region of Turkmenistan. The tree was introduced into Europe at the beginning of the Christian era (Moldenke and Alma 1952). The cultivated varieties of pistachio were introduced into Syria from Turkey and into Italy towards the year 800. Pistachio was introduced into Spain from Italy and France (Lemaister 1959). It was later introduced into the USA in 1853-1854 (Lemaister 1959). Today it is grown as an orchard tree mainly in Iran, the USA, Turkey, Afghanistan, Greece and Syria. Also, it seems that the first commercial plantations were mainly in Iran, Turkey, the US, China and Afghanistan, and other countries adjacent to the wild pistachio nut stands.

1.3.2 : Botany

Pistacia is a genus of the family *Anacardiaceae*, which comprises such widely known trees and shrubs as *Anacardium occidentale*, *Mangifera* spp., *Rhus toxicodendron, R. radicans,* and *R. coriaria* (Chandler 1951; Whitehouse and Stone 1941; Zohary 1952; Ozbek and Ayfer 1958; Davis 1966; Joley 1969; Crane 1984; Ayfer 1963). The most recent monographic study of the genus is that by Zohary (1952), who recognised eleven species of *Pistacia*, of which six are native to Turkey (Davis 1966). Communities of pistachio are very variable, their floristic composition depending on their location which can be steppe-forests, steppe, or semideserts, where beside grasses and wormwoods trees and shrubs also appear from such genera as *Amygdalus, Celtis, Cerasus, Crataegus, Juniperus, Colutea, Rosa,* and *Berberis*. The trees of *Pistacia vera* L. are dioecious, the fruit bud differentiation occuring during the calendar year prior to blossoming (Ayfer 1963). In 1967, Bocconi pointed out the dioecious character in pistachio species and discussed the pollination methods (Whitehouse and Stone 1941). Shoot extension begins at the end of March and terminates between the end of April and the middle of May. Generally 1 or 2 axillary buds located distally on the new growth are vegetative. They are considerably smaller than the inflorescent buds, and may give rise to lateral branches the following year, or they may remain dormant. Inflorescent buds begin expansion at the end of the following March, and anthesis occurs generally in the later part of May and for about three weeks their growth and differentiation is rapid (Ayfer 1963). Thus pistachio bears its fruit laterally on wood produced the previous season (Crane 1984).

Leaves are alternate, deciduous or evergreen, pinnate, more rarely trifoliate or simple. Both staminate and pistillate inflorescences are panicles that may consist of 100 to several hundred individual flowers. Both types of flowers are apetalous, and the pollinating agent is wind. Staminate and pistillate cultivars having similar flowering times must be provided to ensure adequate pollination of the latter. The fruit is a semidry drupe. Its maturity is manifested by a change in the epicarp (skin) from translucent to opaque, and a softening and loosening of the epicarp and mesocarp (hull) from the endocarp (shell) which encloses the embryo (kernel) (Crane and Iwakiri 1980). The endocarp has a thin red-violet fleck and the seeds range in colour from light to dark-green. Endocarp dehiscence is first noted along the ventral suture in late July, about the time ultimate kernel size is attained and progress along both sutures until physiological maturity about the middle of September (Ozbek 1978). Physiological maturity is signalled by easy separation of the epicarp (hull) from the shell (Crane 1984), the equivalent of flesh separation from the pit in a free-stone peach.

1.3.3 : Importance of the species

Pistacia vera L. is a valuable fruit tree and is used for tannins (leaves, galls and bark). As a results of tapping from male individuals a special resin is obtained which is used in the dye industry. The heavy, narrow-ringed and colourful wood of this pistachio is easy to work with and it is also a raw material for the production of high calorie charcoal. Because of its pleasing flavour the nut has recently become widely used in the pastry and ice-cream industries. It is popular with most people at first acquaintance as well as being delicious, so as production increases demand also increases. The pistachio nuts are very nutritious with high oil (58%) and protein (19%) contents, and are relatively low in sugar (7%) depending on cultivars (Bloch and Brekke 1960).

Apart from in its counties of origin, pistachio is also a luxury table nut elsewhere in the world. For human consumption, the pistachio nuts are marketed by quality classes. In general, the smaller the size, the greener the colour (Ayfer 1990). Large nuts with green kernels are very expensive and desirable. Although the taste, aroma and texture in the kernel of small sized cultivars are superior, the bigger size is the more acceptable for the trade. Nowadays, the pistachio industry is of increasing importance especially in Iran, the United States, Turkey and China for the commercial production as table nuts.

Turkey is one of the gene centres of origin of pistachio nut (*Pistacia vera* L.) and its tree is called "Tree of Gold" and pistachio nut is known as "King of Fruits" and "Fruit of Kings" (Ayfer 1990). The most recent monographic study is that by Zohary (1952) who recognised eleven species of genus *Pistacia*, including *P. atlantica, P. cabulica, P. chinensis, P. falcata, P. integerrima, P. khinjuk, P. kurdica, P. mutica, P. Palestine, P. terebinthus. P. vera* is the only species in this genus which produces commercially acceptable edible nuts (Whitehouse 1957, Joley 1979). The leading countries in pistachio nut production are Iran, the United States, Turkey and China, Syria, Greece, Italy, Afghanistan, Tunisia and Jordan respectively, with the world production totalling 3.453.05 metric tons in 1993 (FAO Yearbook 1993). The average annual yield per tree, over 12-15 years of production, ranged from 56.5 kg (fresh weight) for Kerman to 11 kg for Red Aleppo cvs. (Joley 1979). Irrigation practices in pistachio trees increase fruit yield especially in normal yielding years. Irrigation at 20 day intervals produced 57.1 kg/tree during the normal yielding years, whereas without irrigation only 34.5 kg/tree were produced during the same period. During the low yielding periodicity years, the yield from the non-irrigated treatment was 17.3 kg/tree, whereas the irrigation treatment gave a higher fruit yield of 40.3 kg/tree, yet irrigation treatments had no effect on quality of yield (Kanber et al. 1990).

The pistachio tree has been reported to be very xerophilous and gypsocalciphilous (Woodroof 1979, Ayfer 1990). One of most important reasons for the low yield of pistachio nuts in Turkey is that cultivars cannot receive adequate chilling in the places with warm winters (Kaska et al. 1990). The ability of *Pistacia* species to grow in diverse climates and various soil types, generally in the reddish-brown forest soils, is an advantage, and the rootstocks are adaptable to a wide range of soil types (Joley 1979; Woodroof 1979). In Turkey it grows in the reddish-brown rocky soils of the hills with very thin A horizon on sandy soil rich in potassium and magnesium content (Tekin et al. 1990). Beside its commercial use, some of the other *Pistacia* species are used for ornamental planting or as a source of ornament and shade and a wind break for erosion control, because some species such as *P. lentiscus* are evergreen, varying from a low, shrubby bush of 3 to 6 feet in height and 6 to 10 feet in width to a small dense tree 8 to 12 feet in height and 10 to 20 feet in width (Joley 1979). They are also used as a stock on which to graft the commercial cultivars. The outer green shell of the pistachio nuts, *P. vera* L. contains a mixture of phenolic acids (Yalpani and Tyman 1983). Other compositional studies have been reported on the fatty acids, and amino acids of pistachio kernels (Clarke et al. 1976; Beringer and Dampart 1976; Garcia et al. 1992).

One of the outstanding problems in the expansion of the pistachio plantation is the lack of adequate methods of vegetative propagation. The pistachio is the most difficult of all of the nut trees to propagate (Joley 1979). Historically and currently, pistachio is propagated from seed and by grafting buds from elite clones onto rootstocks. This is necessary because pistachio is a natural outbreeder and the resulting seedling populations are heterozygous. Despite significant development of pistachio propagation, the expansion of pistachio plantation is limited by inadequate supplies of nursery material from which to develop uniform, highly productive populations, and by the absence of any constructive breeding programme for the improvement of scion material for such traditional approaches (Barhchi and Alderson 1983). It seems now that these traditional methods for pistachio propagation and improvement, which are costly and time-consuming, and *in vitro* methods since 1982, which are inefficient and unable to produce mass clonal propagation of pistachio trees of improved quality, quantity and yield are not acceptable. Therefore an alternative means for the improvement of pistachio trees is required.

1.3.4 : Conventional methods for pistachio tree propagation and improvement

Due to cross-pollination, commercial pistachio nut trees are virtually as variable as wild populations. The breeding strategy is to exploit this genetic variation using seed orchards and controlled crossing. Currently, in Turkey like elsewhere in the world pistachio trees are

propagated by grafting or budding mature scions onto seedling rootstocks due to difficulties in rooting cuttings. Rootstocks for pistachio are still obtained from seeds, because a successful vegetative propagation method for *Pistacia* species has not yet been found. Rootstocks for pistachio are of two kinds; wild pistachio species which are grafted at the place where they happen to grow, and pistachio seedlings which are used for establishing commercial orchards (Ayfer et al. 1990).

1.3.5 : Propagation by cuttings

Currently, rooting of cuttings is not the most common practice of vegetative propagation in pistachios. However, softwood cuttings of one-year-old *P. chinensis* seedlings treated with 5000 mgl^{-1} IBA resulted in 92% rooting, and no improvement was observed even when higher IBA concentrations (10 000 and 20 000 mgl^{-1}) were used (Pair and Khatamian 1982). Explants taken from *P. vera* seedlings were rooted *in vivo* following a quick dip (5 s) in concentrated IBA solutions; 100 and 77.7% rooting being obtained when 500 and 100 mgl^{-1} IBA were used respectively (Abousalim 1990). As far as rooting of adult material is concerned, rooting was attempted *in vitro* using the quick dip method. Optimal rooting achieved was 50% when the cut ends of four-year-old microcuttings were dipped for 10 s in 1000 mgl^{-1} IBA (Abousalim 1990). Softwood *P. vera* cuttings were successfully rooted under a mist system after very high auxin concentrations were used; 35000 mgl^{-1} IBA was found to be optimal and 88% rooting was obtained after six weeks from planting (Al Barazi and Swabe 1982).

As far as rooting of adult *P. vera* material is concerned, rooting was attempted by several workers (Joley 1960; Joley and Opitz 1971; Sakoury 1976 cited in Bustamante-Garcia 1984) *in vitro* using a mist system. Unfortunately no more than 5% rooting was achieved. Perhaps the major dilemma with rooting cuttings of pistachios is the rapid loss in rooting capacity with increasing age of the parent tree. Ironically, when the time is appropriate to select elite mature trees on the bases of their good past performance, propagation by cuttings is the most difficult problem.

1.3.6 : Propagation by budding

Several *Pistacia* species may be used as rootstocks. The rootstock diameter should be large enough to accommodate pistachio buds which are broad and large as compared with most fruit tree buds (Joley 1979; Woodroof 1979). In the U.S, "Kerman" (pistillate) and "Peters"(staminate) are normally budded onto *P. atlantica* or *P. integerrima* rootstocks (Joley 1979; Crane and

Iwakiri 1981) which have a greater resistance to nematodes and *Verticillum* with than *P. vera*. However, in Turkey, Iran and other Middle East countries, in general, *P. vera* is used as a rootstock for new orchards. Seedlings of this species make more lateral roots and thicker stems than the others and they can reach budding size in a shorter time (Ayfer et al. 1990). However, there are growth differences among seedlings, so between rootstocks and cultivars stock-scion incompatibility may be seen that needs intergrafting. Seedlings of *P. atlantica* and *P. khinjuk* show a rapid increase in length, but make thinner seedlings than the others so they reach budding size later. However, there is no stock-scion incompatibility between cultivated varieties and these rootstocks (Ayfer et al. 1990).

Seedlings of *P. atlantica* and *P. terebinthus* are widely used for commercial production (Joley and Opitz 1971; Woodroof 1979). Top worked cultivars on these two rootstocks quickly outgrow and outyield those grafted onto *P. vera* in spite of the characteristically slow growth in the nursery of the two former rootstocks (Joley 1979). *P. atlantica* and *P. terebinthus* have been reported to be highly susceptible to *Verticillium*. Because of very its slow growth habit, *P. terebinthus* when grafted at the place where it grows, makes dwarf trees and begins to produce early, large and quality fruits. It is generally accepted that *P. terebinthus* is one of the most valuable rootstocks and together with *P. vera* can be a suitable rootstock for intensive cultivation under irrigation conditions (Ayfer et al. 1990). Concerning root knot nematode susceptibility, seedling progeny of *P. vera* x P. *atlantica*, *P. vera* x *P. interregima* have been found to be highly resistant as compared to *P. vera* x *P. terebinthus* seedling, which proved to be the least resistant (Joley and Whitehouse 1953). However, *P. terebinthus* was reported to be tolerant to *Phytophthora* spp (Pontikis 1977).

In Turkish pistachio-growing regions 'budding' is the most common propagation technique. This technique can only be exercised during a very short period of the year (Kaska et al. 1990). The outlook seems to be that traditional methods of vegetative propagation in pistachios still suffer from many problems. A potential solution to these problems would be the development of methods for vegetatively propagating the rootstocks. It would also be advantageous, for example, to propagate only preferred rootstocks which impart a low production of non-bearing trees while maintaining the other superior characteristics associated with the rootstocks (Bustamante-Garcia 1984). Unfortunately, attempts to propagate rootstocks vegetatively by conventional methods of soft and hard wood cuttings have given inconsistent results (Sakoury 1976; Joley and Opitz 1979; Al Barazi and Schwabe 1982). The low efficiency and difficulties in propagating mature trees are the most important problems. Consumption of pistachio nuts is greater today than ever before and population increases will ensure a rising annual consumption. World production has increased significantly over the past 20 years mainly due to

the growing demand in the world markets and the future appears more optimistic for the pistachio nut. What is more, Southern Anatolia Project (GAP) is now going to create an irrigated land opportunity for the pistachio nut (Ayfer et al. 1990). The pistachio production is expected to increase (286%) after the completion of GAP (Mart and Karaat 1990). Thus, plant protection is expected to be a widespread and abundant problem. Therefore, there is an urgent need for large numbers of improved, fast growing trees to establish the new pistachio orchards. The traditional methods used for pistachio propagation and improvement are apparently not adequate to meet these demands. So , new techniques to supplement the traditional methods must be developed. Plant tissue, organ and cell culture techniques provide a promising and alternative approach to the traditional methods.

1.3.7 : Plant tissue, cell and organ culture techniques

The term plant cell culture covers a wide range of techniques involving the culture of explants, i.e. cells, tissues or organ from plants, generally under aseptic conditions, in or on a sterile growth medium typically containing sugar(s) as an energy and carbon source, mineral salts, and growth substances, sometimes solidified, e.g. with agar.

Techniques of plant cell culture broadly categorised are as follows:

1. *Organ cultures*
   a- root culture
   b- shoot (apical) culture
   c- anther and pollen culture
2. *Tissue cultures (callus)*
3. *Cell cultures (cell suspensions and protoplasts)*

For the purpose of clonal propagation, system 1 is preferred, since meristem or shoot derived cultures are generally believed to be more genetically stable.

Cell culture, because of the possibilities it offers for the control of environmental and nutrient conditions, is important in research into e.g. cell cycle, cell growth and development and the totipotency of plant cells. It also has important practical applications. Practical applications of plant organ, tissue and cell cultures are summarised as follows (Yeoman, 1993):

- Shoot tip cultures for production of virus-free stock plants e.g orchids
- Rapid propagation of stock to produce a clonal population, e.g. strawberries
- Propagation when normal macro-technology is not available, e.g. oil palm, coconut, and palm
- Storage and preservation of plants with a poor seed viability

- Production of homozygous plants for plant breeding using anther culture, e.g. wheat in China.

Micropropagation is one of the major areas of plant cell biotechnology which is of commercial importance. It refers to the rapid vegetative propagation of a plant using tissue culture technology, and usually involves conditions not obtainable in nature (Tisserat 1981). In micropropagation, shoot tips and axillary buds may be cultured under conditions which promote the growth of a mass of proliferating lateral shoots which, after 4-8 weeks, may be separated and subcultured. Rooting may be induced by transfer to a suitable medium. The use of shoot apex cultures for the rapid clonal propagation of plant material was first carried out by Morel (1960). Alternatively, a callus or suspension culture may be established, subcultured and eventually induced to form plants. This method is especially useful for the rapid multiplication of pathogen-free, heterozygous and ornamental plants.

According to Yeoman (personal communication) advantages of micropropagation are that it:

- ensures faster cloning of scarce plant material for evaluation trials or for the early establishment of clonal material
- is important for the multiplication of infertile plant hybrids, for example orchids, or for highly heterozygous variable species, such as pistachio, for which clonal propagation provides a means of providing a uniform plant population
- ensures a supply of disease-free stock for plant breeding
- removes seasonal constraints, so that micropropagation may be carried out throughout the year
- ensures multiplication of plants with a desired genotype
- enables a mass propagation with a rapid turnover of biomass and induction of genetic variability for the production of the pistachio trees which are high yielding and resistant to pests and diseases
- enables the multiplication and release of new varieties on to the market more rapidly than by conventional seed methods
- can be performed in a small space because small explants are multiplied
- requires minimal attention between subcultures (e.g. watering, weeding, spraying etc.).
- may be used *in vitro* for stock plants
- may be possible to automate propagation procedures for some species, so reducing labour requirements
- may be used to clone engineered parent lines as an intermediate stage.

1.3.7.1 : Micropropagation of pistachio trees

Although pistachio nut trees have been cultivated for centuries, special attention has been given to *in vitro* culture of this species since 1982. Two resumes of all the *in vitro* work carried out with pistachio trees over the period 1982-1989 were published by Hansman and Owens (1986) and by Barghchi and Alderson (1989). This is summarised in Table 1. Most of the effort put into the micropropagation of *Pistacia* species has been directed towards manipulating the regeneration process of cultured tissues, in particular using juvenile and/or adolescent plant material or seedlings of commercial cultivars through surface sterilisation of pistachio seeds, the appropriate choice of a nutrient medium, selection of suitable explant and control of the physical culture environment.

The theme of this study will therefore be the organogenesis and asexual embryogenesis of mature *Pistacia* species. The forthcoming subsections will concentrate exclusively on the literature relating to *Pistacia* species. However, where the literature does not provide the required information, especially on embryogenesis, I will refer to studies carried out, in particular, with other woody species.

1.3.8 : Choice of nutrient medium

Micropropagation of pistachios has been generally performed on Murashige and Skoog culture medium for establishment on shoot multiplication (Barghchi 1982; Martinelli 1988 and Abousalim 1990). Barghchi (1985) tested MS medium against Lloyd and McCown (WPM) formulae and found no difference between the performances of shoots of *P. vera* when grown on either of the two media. The response of pistachio tissues to 6 different basal media (G5, Cheng, modified Erikson, Heller, White and Woody Plant) in shoot proliferation was tested with the finding that the best response in terms of the number of nodes producing shoot, was obtained with a modified Erikson liquid medium using filter paper bridges (Bustamante-Garcia 1984). However, a comparable study was performed using the four different media in the stage of shoot multiplication (Abousalim 1990). MS medium proved superior for *P. vera* shoot multiplication when compared with the formulae of Anderson, WPM and Knop.

The nutrient media usually employed to induce roots obtained in pistachios *in vitro* have a reduced mineral salt content (1/2 strength of the mineral salt) with a lower sucrose level (Barghchi 1982 and Abousalim 1990). A comparison of the ionic strength of the tested media showed that MS has a relatively high mineral salt concentration as compared to the ionic

composed of the WPM, A and K media. The MS medium induced more callus, more faintly necrotic leaves and generally supported the best growth of pistachio *in vitro* in comparison with other media formulations used widely for tissue culture of tree species (Abousalim 1990). Medium modifications for the successful micropropagation of pistachio were studied by Parfitt and Almendi (1994). From the basal media tested, DKW medium was selected. K, N, B and Zn concentration were adjusted to provide optimum growth and multiplication of shoots, some of which were grown and multiplied for 2 years. They also tested the use of TDZ as a growth regulator, but it was not beneficial.

Table 1 : The regeneration of pistachios *in vitro* since 1982, with examples of the parts and ages of the species used as explants.

Species	Explant & (age)	Reference
P. vera	Shoot tip (juvenile)	Barghchi (1982)
	Nodal axillary/apical bud segments (mature)	Barghchi (1985)
	Axillary bud segment (juvenile)	Barghchi and Alderson (1983a,b)
	Shoot tip and axillary bud	Barghchi and Alderson (1985)
	Meristem tip (mature)	Barghchi and Martinelli (1984)
	Axillary and nodal bud segments (juvenile and mature)	Bustamante-Garcia (1984)
	Shoot tip (juvenile)	Yucel et al. (1991)
	Shoot tip and axillary bud (juvenile and mature)	Abousalim (1990)
P. atlantica	Nodal segments (juvenile)	Martinelli (1988)
	Nodal bud segments (juvenile)	Abousalim (1990)
P. integerrima	Nodal segments (juvenile)	Martinelli (1988)
P. terebinthus	Shoot tip (mature)	Pontikis (1984)
P. khinjuk	Shoot tip (juvenile)	Barghchi (1986)
P. mutica	Shoot tip (juvenile)	Barghchi (1986)
P. palaestina	Shoot tip (juvenile)	Barghchi (1986)

1.3.9 : The physical culture environment

Other variables in the culture environment that can influence growth and development are light and temperature. It is clear from a consideration of the literature that the physical aspects of the culture environment have not as yet been critically evaluated, and most reports on the culture of pistachios are deficient in this respect. The effectiveness of temperature on shoot growth and proliferation of pistachio explants have been reported, but no significant differences in

proliferation and growth of *P. vera* seedling material were found between cultures incubated at 20°C and those grown at 25 ± 2°C (Barghchi 1985 and Abousalim 1990). However, the second researcher found that growth and proliferation was affected by the temperature at which cultures were incubated, with significantly better performances being obtained at 25°C as opposed to 19° C. Combined temperature/photoperiod treatments also affected the responses of four-year-old *P. vera* derived shoots. Improvement of shoot growth and proliferation, although not significant except for fresh weight, was observed at 29°C/16 h (Abousalim 1990).

1.3.10 : Clonal Propagation

Vegetative propagation of pistachio trees by tissue culture methods of micropropagation may be achieved by two approaches, namely by (a) organogenesis and (b) somatic (non zygotic) embryogenesis. The first approach leads to plantlet formation via organogenesis through the production of unipolar shoots, which must then be rooted in a multi-staged process. However this method has an encouraging value with pistachio trees. In contrast, there is no report on somatic embryogenesis which leads to the formation of a bipolar embryo, through steps that are often similar to zygotic embryogenesis.

1.3.10.1 : Micropropagation via organogenesis

This is a major route consisting of at least four distinct stages, namely (1) culture establishment and/or bud induction, (2) shoot development and multiplication, (3) rooting of developed shoots and (4) weaning of plantlets. In some cases stages (3) and (4) are combined, particularly when rooting is carried out *ex vitro* (Thorpe *et al.* 1990).

1.3.10.2 : Culture establishment

Shoot tips and nodal bud segments were the main explant types used for culture establishment (Barghchi 1982; Pontikis 1985; Abousalim 1990). Initiating cultures with a decontaminated plant material is important to the success of the *in vitro* propagation.

Surface sterilisation of pistachio seeds and one to two-year old greenhouse-grown plants is reported to be achieved in two steps: first in 70% ethanol for 45 s and then in 20% commercial bleach solution (sodium hypocholorite) for 25 min (Barghchi 1982). In the case of mature plant materials, it was difficult to disinfect and high levels of contamination have been observed in

nodal bud segments (Barghchi and Martinelli 1984). Surface sterilisation of greenhouse-grown plants was achieved by giving them a 5 min wash in detergent followed by a 5 second dip in 70% ethanol and a 5 min soak in 20% Chlorox (Bustamante-Garcia 1984). However, data on the effectiveness of the sterilisation method have not yet been published.

Surface sterilisation of pistachio seeds was achieved using 20% NaOCl solution supplemented with 0.1% Tween 20 as a wetting agent using a shaker or a vacuum infiltration system (Abousalim 1990). The use of a vacuum to aid surface sterilisation of pistachio seeds was efficient in preventing fungal contamination. No fungal contamination was observed in the case of the vacuum treated seeds and all the infections observed were of bacterial origin. In the case of the shaker treated seeds, 13.5 and 2.7% fungal contamination were observed when 12 and 20% bleach were used, respectively (Abousalim 1990).

Establishment of adolescent (up to two year) material was low at the first stage of culture initiation mainly because of severe browning. Improvement has been made by transferring the explant to new sites on the medium within the same vessel once browning was observed (Barghchi 1986). Growing up to four year-old mother plants in the glasshouse, severely pruning them and frequently subculturing the cultured explants has been reported to give good results (Martinelli 1984). The establishment of mature fruit bearing trees has been difficult because of severe browning (Barghchi 1985). Pre-soaking of explants in malonic acid 10-100 mgl^{-1} to the culture medium only marginally delayed browning. More suitable material for subculture appeared to be provided by conditioned mother plants, i.e. pruned, repeatedly grafted, sprayed with BAP and GA_3 or from micrografting (Barghchi and Martinelli 1984; Barghchi 1985). However, poor establishment was observed when explants of mother plants sprayed with GA_3 were used, and maintenance of shoots which resulted from *in vitro* micro grafting-tips onto juvenile rootstocks was difficult (Barghchi 1985). Nodal explants from adult *P. vera* and *P. atlantica* trees were found to be unresponsive to *in vitro* initiation (Bustamante-Garcia 1984). Rejuvenation of adult *P. vera* material was attempted by GA_3 sprays, by grafting onto juvenile seedlings and by inducing the formation of spheroblast, with success utilising the GA_3 at 250, 500, and 1000 ppm induced terminal buds and shoots with juvenile leaf morphology. Nodal bud segments from these shoots responded favourably to *in vitro* initiation (Bustamante-Garcia 1984).

Surface sterilisation of four-year old *P. vera* grafted actively growing shoots under protected glasshouse conditions and shoots derived from 30-year-old field-grown *P. vera* trees with 20% bleach solution containing 0.1% Tween 20 for 20 min using a shaker and incubating cultures

during the first stages of culture initiation in darkness for up to three weeks would seem to be satisfactory in limiting media browning and tissue injuries and in producing good establishment results (Abousalim 1990). Following the above procedure, up to 93% survival was achieved using four-year old derived explants. Although a high survival (85%) was obtained with explants derived from 30-year old trees, maintaining cultures was difficult because of a slow decline in growth and increase in vitrification and chlorisis (Abousalim 1990).

1.3.10.3 : Shoot multiplication

Vegetative propagation of *Pistacia* species is mainly achieved by inducing growth and development of multiple shoots from pre-existing apical and axillary buds. Multiple shoot proliferation was better with BAP than with kinetin (Barghchi 1982) and 4 mgl^{-1} BAP was optimal for shoot proliferation of *P. vera* seedling material (Barghchi 1982; Abousalim 1990). Following subculturing on medium containing 4 mgl^{-1} BAP up to 40 and 35 shoots were obtained per culture (Barghchi 1982). Lower BAP concentrations have been used by Martinelli (1988) to propagate one - to four-year-old *P. vera* plant material. Lower concentrations of BAP have also been used in the case of the four-month to four-year-old *P. atlantica* (0.7 mgl^{-1}), *P. integerrima* (1 mgl^{-1}) seedling rootstocks and mature *P. terebinthus* (2.5 mgl^{-1}) (Pontikis 1985).

The addition of as little as 0.05 mgl^{-1} NAA to the culture medium inhibited seedling explant growth (Barghchi and Alderson 1985) and higher concentrations increased callus growth at the base of *P. vera* seedling shoots (Bargchi and Alderson 1983). Shoot growth and proliferation of seedling material was not improved by adding 0.25-4 mgl^{-1} GA_3 or GA_{4+7} to the culture medium containing 4 mgl^{-1} BAP (Barghchi and Alderson (1983). In the case of *P. atlantica* seedling materials BAP at 1 mgl^{-1} proved optimal. Production of shoots with optimum conditions for subculturing of their nodes can be achieved by transferring small shoots with their accompanying original node and callus tip fresh to medium supplemented with BAP+GA_3+IBA, every two weeks (Bustamante-Garcia 1984).

The continuous use of optimal levels of BAP produced dwarfed shoots with very small leaves and this type of material proved inappropriate for subsequent sub-culturing as well as for rooting (Abousalim 1990). The same worker also found that the use of low concentrations of 2iP in a mixture with sub-optimal concentrations of BAP was beneficial in improving the growth and qualities of shoots without significantly affecting shoot multiplication at the tested levels. A mixture of 0.5 mgl^{-1} 2iP and 1.5 mgl^{-1} BAP proved optimal.

1.3.10.4 : Rooting and hardening of plantlets

These processes may be carried out separately or together depending on whether rooting is carried out *in vitro* or *ex vitro*. Rooting has been easily achieved both *in vitro* (Barghchi and Alderson 1983; Pontikis 1985; Martinelli 1988; Abousalim 1990) and *in vivo* (Martinelli 1988; Abousalim 1990).

1.3.10.5 : *In vitro* rooting

In vitro rooting has usually been done using agar-solidified medium as the substrate. IBA was found to be the most suitable auxin for *in vitro* rooting. Rooting was improved by using half strength macro nutrients during the root induction stages, by incubating cultures under dark conditions for the first seven days of culture on root initiation medium and finally by transferring shoots to an auxin-free medium after the first roots reached 1 to 2 mm length (Barghchi 1982). However, rooting was improved in the case of *P. vera* seedling materials (83%) by keeping the levels of MS micro nutrients at their full strength (Abousalim 1990). No significant differences in rooting were observed with either 2.5, 3.0 or 3.5 mgl^{-1} IBA, and 80% rooting with three roots per shoot was obtained with up to two year old *P. vera* seedling derived material (Barghchi and Alderson 1985). However, optimal rooting responses were achieved with explants subcultured once on multiplication medium and induced to root on 2 mgl^{-1} and 1 mgl^{-1} IBA, respectively (Abousalim 1990). Rooting of the original explants was induced with both NAA and IBA solid or liquid (Bustamante-Garcia 1984). However, the best responses were obtained with 1 mgl^{-1} NAA using liquid medium and filter paper bridges. Rooting of the tissue culture derived explants was low (25%) and only occurred in explants with leaves and the mechanism by which light inhibits rooting appears to consists of a combination of factors including accumulation of p-coumaric acid, IAA, and other factors which inhibit high levels of the IAA oxidation products at the cut end of the cuttings (Bustamante-Garcia 1984). Poorer rooting was obtained when explants were taken from decapitated mature *P. terebinthus* (60%) (Pontikis 1985), and from up to four-year-old grafted *P. vera* plants (30-40 %) (Martinelli 1988). In the case of four-year old *P. vera* derived shoots, optimal rooting (50%) was achieved when the cut ends of micro cuttings were dipped for 10 s in 100 mgl^{-1} IBA (Abousalim 1990). Rooting of *P. integerrima* micro cuttings taken from up to four-year-old seedlings was achieved within three weeks (Martinelli 1988). *In vitro* rooted shoots were successfully established (70-80%) in peat-based compost in spite of the dark colour of roots. One root per explant was sufficient to ensure establishment (Barghchi and Alderson 1985). However, other experiments in which *in vitro* rooted *P. vera* shoots have been investigated,

weaned plantlets failed to grow after being transplanted into glasshouse conditions (Martinelli 1988). Artificial inoculation of *in vitro* rooted *P. integerrima* explants with vesicular-arboscular mycorrhizae fungi was also investigated. Significant increases in plant growth of inoculated plantlets were obtained when unaffected substrates were used (Martinelli 1988). In studies with other woody species, elevated sucrose (40 gl^{-1}) increased stem signification and acclimatisation success (Driver and Suttle 1987). *In vitro* rooted shoots derived from *P. vera* seedling materials were successfully established *in vitro* with 81.8% being achieved (Abousalim 1990).

1.3.10.6 : Micropropagation via somatic embryogenesis

1.3.10.6.1 : General considerations

In the case of pistachio, no previous attempt appears to have been made to induce somatic embryogenesis. Plantlet regeneration via this route is preferred, wherever possible, because of (1) the difficulty and time consumption with the rooting of shoots derived from adventitious budding, (2) somatic embryogenesis provides an effective method for rapid propagation of large number of plants, and (3) embryonic suspensions obtained from embryonic callus can serve as a source of embryogenic protoplasts that can be used for genetic engineering of trees (Thorpe et al. 1990). Several advantages of this method have been noted: (a) it represents a method of obtaining true rejuvenation from mature trees, (b) costs are reduced when compared to plantlet production via adventitious budding, (c) problems associated with field performance of plantlets will be eradicated, and large number of embryos can be stored relatively easily in liquid cultures (Boulay 1987).

For the first time Steward et al. (1958) and Reinert (1958) reported that embryos could be grown from carrot; *Daucus carota*, in cell culture. These were eventually termed somatic embryos because they developed directly from somatic cells without gametogenesis and synogamy embryos (rather than embryoids) because they are structurally and biochemically identical to zygotic embryos (Ammirato 1987). While the list of woody species that can be propagated through this method has been increasing, the percentage of recovery of plants still remains relatively low.

1.3.10.6.2 : Woody plants exhibiting somatic embryogenesis

Many articles concerning adventive (or asexual, secondary etc.) embryogenesis in tissue culture of woody plants such as forest trees, both conifers, and angiosperms have been published in recent review articles and books (Von Arnold and Sand Wallin 1988; Tulecke 1987; Ammirato 1984; Litz and Jaiswal 1991; Thorpe et al 1990; Mengoli and Bagni 1992; Ahuja 1992; Attree and Fowke 1993; Jain et al. 1995a, 1995b, and 1995c).

1.3.10.7 : Development of an embryonic system

1.3.10.7.1 : Culture initiation

Generally reports of initiation of embryonic cultures are based on the use of immature and mature zygotic embryos. Diploid cultures of conifers are most readily initiated from zygotic embryos, which are generally dissimilar from each other. Low osmotic conditions, usually provided by 1-3% sucrose, are beneficial for induction for conifers. The results from Association Foret-Cellulose laboratories and recent literature indicate that success in ESM initiation is fully dependent on the chronological age of the sporophyte, the source of EMS (hypocotyls and cotyledons), and on the genotype (Ruaud et al. 1992; Park et al. 1993 and Mo et al. 1989). The auxin 2,4-dichlorophenoxyacetic acid (2,4-D) generally has been the preferred auxin used for the initiation of ESM of most conifer species (Gupta et al. 1991, Tautorus et al. 1991). Naphthaleneacetic acid (NAA) has also been used for the ESM induction of *Picea abies* (Verhagen and Wann 1989). Cytokinin is important, and is usually included with an auxin at different concentrations (Attree and Fowke 1993). Generally spruces and larches have yielded embryonic tissues more readily than pines. Induction from pine zygotic embryos was improved by cocultivation with the megagametophyte, which may initially supply some of the PGRs required for induction (Attree and Fowke 1993). In such instances, both an auxin and a cytokinin must subsequently be supplied for continued proliferation of the pine somatic embryos (Becwar et al. 1990). Initiation from pines has generally been easier using immature embryo explants, but some conifers, such as *Pinus banksiana* Lamb (Tautorus et al. 1991), have remained unresponsive to *in vitro* embryogenic techniques despite much effort. Genotype specificity for embryogenic induction occurs in conifers (Attree and Fowke 1991). For example, the ability of white spruce to undergo induction was significantly affected by the seed provenance's (Tremblay 1990), and significant differences in response to induction among open-pollinated families of black spruce were also observed (Cheliak and Klimaszewska 1991). Additionally, the induction of

somatic embryogenesis in white spruce is under strong additive genetic control (Park et al. 1993).

Embryogenic mass induction using explants from mature trees has not been conclusively demonstrated (Attree and Fowke 1993). Although no data are available, it is likely somatic mutations will not be evident following *in vitro* propagation of conifers via somatic embryogenesis, due to the general lack of a green-non-embryonic callus phase. Induction of EMS has mostly been done in the dark but it has also been reported to occur in the light (Verhagen and Wann, 1988).

1.3.10.7.2 : Explant sources

Initiating the development program for somatic embryogenesis from a cell or a group of cells frequently depends on the nature of the explant's source. Juvenile tissues of certain species appear to be the most suitable for induction of somatic embryogenesis (Tulecke 1987). The characteristics of juvenile and adult tissues have been discussed by Bonga (1981) and Durzan (1982). Among the tissues used to induce somatic embryos are the cotyledons, ovule, leaf, immature or mature embryos, hypocotly and cell suspensions as well as anthers, pollen, endosperm and nucellus (Carron and Enjalric 1985). In angiosperms, various explants have been used for generating embryonic callus, but in conifers success has been achieved mainly with immature and mature embryos (Thorpe et al. 1990; Von Arnold 1987; Attree et al 1987; Gupta and Durzan 1986).

1.3.10.7.3 : Effects of the medium

The nutritional requirements for somatic embryogenesis are not understood. In general, media used contain high levels of salts and both NH_4^+ and NO_3^- (Thorpe 1988). They are neither specific nor exclusive, since various responses produce results. The most commonly used medium is that of MS either in its original form or modified, but others work as well or better for specific tissues. Media requirements for the initiation of embryogenic cultures do not appear to be very specific. There are several basal media (especially modified MS) which have been used for the initiation of embryogenic cultures (Jain et al 1995a).

1.3.10.7.4 : Effect of the plant growth regulators

Culture initiation has been carried out with different concentrations of hormones. Auxins, usually 2,4-D or NAA and in some cases a cytokinin, are needed. Studies on the effect of these substances on embryogenesis are reported (Debruijne et al 1974; Zhan 1983; Attree and Fowke 1993).

1.3.10.7.5 : Inhibitors and promoters

Treatments that promote normal maturation appear to establish, first, a balance between 'inhibitors' such as ethylene, ethanol, gibberellic acid (GA), auxin, phenolics, tannins, polyamines, ABA and carbon dioxide, and 'promoters' such as dimetylamino succinic acid, ethephon, ABA and inhibitors of auxin synthesis have been reported to stimulate embryogenesis. Treatments with ABA reduced the total number of embryos and abnormal embryo formation (Kamada and Harada 1981). Additionally factors which were correlated with increased embryogenesis were low levels of sucrose, irradiation and charcoal.

1.3.10.7.6 : Culture maintenance

Several methods are currently used to support continued development of the EMS. Once an embryonic line is established it must be maintained in culture without loss of morphogenetic competence. Embryonic cultures are usually maintained on a medium similar to the induction medium, which typically includes an auxin and a cytokinin but with lower level concentrations of them when compared with media used for initiation (Durzan and Gupta 1988). Cultures initiated from immature *Picea glauca* embryos showed a loss of 50-75 percent of embryogenic potential in a few months (Webb et al. 1989). A 60 percent loss of embryogenic potential was reported for cultures from mature embryos of *P. sitchensis* (von Arnold and Woodward 1988). Cultures of EMS have been maintained in liquid medium using 250 ml Erlenmeyer flasks, continuously rotated at 100-120 rpm in the dark and sub-cultured at approximately 7 day intervals (Gupta and Durzan 1987). Maintenance of EMS has also been done by transferring cultures on solid medium every 10-12 days onto fresh medium (Jain et al. 1995a). Liquid culture maintenance requires close control of culture density. Changes in embryogenic potential of EMS cultures and their growth has been reported after two months of encapsulation (Onay et al. 1995b). Differences in yield and morhology of somatic embryos on development and maturation media have described to variation in embryogenic potential of EMS during liquid cultures in bioreactor (Lulsdorf et al. 1992).

Liquid culture is more suitable for maintaining conifer cultures in a rapidly growing state for large-scale propagation (Attree and Fowke 1993). Mechanically stirred or airlift bioreactors are the ideal vessels for providing uniform growth in liquid culture (Tautorus et al. 1991). Liquid cultures maintained in flasks are capable of sustained rapid proliferation, but are prone to swift changes in embryonic potential and vitality, so a reserve supply of somatic embryos is required to reestablish the liquid culture should it fail (Attree and Fowke 1993). These stationary cultures should be sub-cultured every 2-4 weeks to prevent browning and death.

For scaled-up applications, the maintenance of reserve cultures could be time consuming and costly, especially where large numbers of genotypes are required for clonal forestry. However, Joy et al. (1991) reported that stationary cultures could be maintained in sealed flasks for up to a year without subculturing. It is also possible to slow the growth of reserve cultures by maintaining them at low temperatures i.e. 0-10°C (Attree and Fowke unpubl. cited in Attree and Fowke 1993). Cryopreservation of immature somatic embryos in liquid nitrogen is the best method for long-term preservation, so can be used to preserve genotypes while extended field tests are carried out (Attree and Fowke 1993). Elite genotypes could then be removed from cryogenic storage and cultured on solidified medium, then bulked up in liquid suspensions for mass propagation.

1.3.10.7.7 : Embryo development and maturation

In order to encourage the production of mature developmental stages of conifer somatic embryos, immature somatic embryos must be transferred from an environment that promotes cleavage polyembryogenesis (auxin, cytokinin, and low osmotic concentration), to one containing ABA and ideally a raised osmotic concentration (Attree and Fowke 1993). Early-stage embryo head size is important for cotyledonary embryo development. Smaller early-stage embryos often do not develop cotyledonary embryos on development media (Gupta et al. 1993). Gupta and Pullman (1990) have described the application of increased osmolality, or water stress, during early-stage embryogenesis. Increasing osmolality (to 180-250 mm/kg) by applying osmoticants at early stages enlarges the embryo's head size. Without this treatment, most loblolly pine and Douglas fir genotypes form early-stage embryos with small heads. These eventually lead to abnormal cotyledonary embryos or do not continue development. Immature somatic embryos may be transferred to a medium containing activated charcoal and then exposed to ABA (Becwar et al. 1987) or directly transferred to ABA-containing medium for several subcultures (Durzan and Gupta; 1987; von Arnold and Hackman 1988). ABA has also been used with indole-butyric acid (IBA, 0.1-1 ppm) for development of Norway spruce somatic embryos (Becwar et al. 1987).

I) Effects of abscisic acid

Abscisic acid exists in different enantiomers (-), (+) or racemic (±) forms; however, Dunstan et al. (1992) observed that only the (+) form was effective in promoting maturation, and was itself rapidly converted by the cultures to phaseic acid. Effective maturation, therefore, depends upon the ratio of (+) to (-) enantiomers (Attree and Fowke 1993). Abscisic acid (ABA) plays several roles in conifer embryo development. In general, ABA accumulates during mid-to-stages of seed development and prevents the developing embryo from germinating precociously (Kermode 1990). The activation of genes responsive to ABA (rab genes) leads to the accumulation of mRNAs controlling, among other processes, the accumulation of storage products such as proteins, lipids and starch, and also the late embryogenesis abundant proteins thought to be involved in the acquisition of desiccation tolerance (Skriver & Mundy 1990). Physiological and biochemical studies concerning ABA during maturation of conifer zygotic embryos and seeds are currently scant. Nontheless, cleavage polyembryony is inhibited by ABA, allowing embryo singulation and continued growth of individual embryos (Gupta et al. 1991). In early studies, maturation of conifer somatic embryos was attempted by culturing immature embryos on medium containing reduced or no PGRs (Hakman et al. 1985; Hakman and Fowke 1987; Lu & Thorpe 1987), but maturation was infrequent, and the few recovered plantlets were abnormal by present standards. Researchers have used charcoal to absorb the growth regulators before repeated transfer to development and maturation media with ABA.

Complex carbohydrates have also been combined with ABA for improved embryo development and maturation. For example, 6 percent sucrose and ABA were useful for loblolly and white pines (Finer et al. 1989), and maltose, lactose and starch for white fir embryo development (Schuller and Reuther, 1989). Further factors may be causing the disparity in optimal ABA concentrations reported for embryo maturation. For example, Afele et al. (1992) noted that for blue spruce the auxin to cytokinin concentrations used for induction influenced the subsequent concentration of ABA required for optimal maturation frequency. Thus, a low auxin to cytokinin ratio during induction required a higher ABA concentration for maturation (Attree and Fowke 1993).

Further embryo development often occurs with the removal of ABA by activated charcoal (Durzan and Gupta 1987) or transfer to medium free of ABA (Boulay et al. 1988). Embryo development has been obtained on a semisolid medium or on artificial supports such as filter paper, cheesecloth or fabric saturated with liquid medium (Gupta and Durzan 1986; Boulay et al. 1986; von Arnold and Hakman 1988). The actual role of ABA and phaseic acid in promoting the

maturation of conifer somatic embryos remains unclear. In general, the optimal ABA concentration required to promote maturation of conifer somatic embryos needs to be determined empirically for each genotype of a species for a particular culture regime (Attree and Fowke 1993).

II) Effects of osmoticum

The type of osmoticum used in conjunction with ABA profoundly affects the development of conifer somatic embryos (Attree and Fowke 1993). Thus, simple sugars and salts were not always as effective as high molecular weight osmotica such as polyethylene glycols (PEGs) or dextrans. Increased osmolality in the development and maturation medium inhibits embryo greening and precocious germination, allowing the embryo to mature further (Gupta et al. 1993). According to Attree et al. (1991), low molecular weight compounds cross the cell membranes and cause water to be withdrawn from the protoplast by osmosis, leading to plasmolysis. Such absorption leads to adjustment of tissue osmotic potential and deplasmolysis (Attree and Fowke 1993). In contrast, high molecular weight compounds simulate a non-osmotic moisture stress at the cellular level, because the large molecular size of the solute excludes its passage through plant cell membranes, so preventing entry into cells and causes plasmolysis (Attree and Fowke 1993). The ineffectiveness of high concentrations of low molecular weight osmotica to promote maturation of conifer zygotic and somatic embryos is illustrated by Gates and Greenwood (1991), Roberts (1991).

Additionally, Gupta and Pullman (1991) used increased osmolality, together with ABA and charcoal, in the development and maturation of conifer embryos and produced good quality cotyledonary embryos from elite Douglas fir genotypes via a combination of osmolality (ranging from 350-600 mm/kg) ABA (2.5-100 ppm) and charcoal (0.05-025 percent). Cornu and Geoffrion (1990) were the first to report that PEG 6000 encouraged maturation of larch somatic embryos in liquid medium, but subsequent plant development and rooting was poor. Embryo development media often contain reduced nitrogen in the form of amino acids such as glutamine, asparagine, and argine (Boulay et al. 1988). Gelrite, a bacterial gellan gum used as a gelling agent, has been reported to produce more embryos than agar (Hakman and von Arnold 1988).

1.3.10.7.8 : Germination

Seed germination begins with imbition and ends with the start of elongation, usually of the radicle. Embryo drying may activate genes for germination and eliminate residual mRNA which

was required availability upon hydration, for translation of the proteins required for germination (Attree and Fowke 1993). Such events were recorded for *Pinus concartya* (Dougl.) zygotic embryos (Gifford et al. 1991). Similar events have not yet been described in conifer somatic embryos but partially or fully dried somatic embryos show good synchronisation of root and shoot elongation (Attree and Fowke 1993). Poor post germinative vigour of somatic embryos may be due in part to a failure to completely break dormancy (Gray & Purohit 1991). Seeds of many plants commonly require stratification for several weeks or months at a specific temperature to be released from dormancy (Whitemore 1991; Kermode 1990). It has been determined that germination of *Picea abies* cotyledonary embryos is better in dark than in light (von Arnold and Hakman 1988). The germination rate of selected cotyledonary embryos varies between 35-90 percent (von Arnold and Hakman 1988, Becwar et al. 1988, Gupta et al 1993). Germination rate of conifer embryos is strongly related to the storage protein content of the embryo (Roberts et al. 1991). This can be influenced by a high relative humidity treatment (Webster et al. 1990) that avoids the rapid depletion of major storage proteins observed when somatic embryos of *Picea sitchensis* are directly transferred from maturation conditions onto a growth regulator-free medium (Roberts et al. 1991). Selection of desirable mature somatic embryos is a vital area for automation in industrial applications (Gupta et. al 1993). Moreover, automation may be used to select and transfer desirable *in vitro* germinated plantlets to soil (Attree and Fowke 1993).

1.3.10.7.9 : Weaning

Weaning is very important for the future *ex vitro* plant development. The success of weaning is dependent on the four main factors (Paques et al. 1995): a) initial vigour of plantlets, photoperiod length, c) relative humidity, and d) soil composition. A 90% success in weaning is possible by decreasing the relative humidity from 90% to 50% over a 2 month-period when a mixture of non-composted bark and yellow peat (3/1) was used for hardening for the plantlets of *P. abies* (Paques et al. 1995).

1.3.10.7.10 : Field performance

Until now little data is available on nursery and field trials with somatic embryo derived plantlets. The first report on the field performance of the plantlets was by Becwar et al. (1989) who reported that 29% of *Picea abies* emblings were successfully established in soil. A field trial of 1200 *Picea glauca engelmannii* plantlets from 71 genotypes have been established in soil by Roberts et al. (1993). At the end of the first season in the nursery, plantlet survival varied from 80-100% and was genotype dependent. Until now, somatic embryo derived plantlet development

appears normal and no somaclonal variation has been reported in conifer using chromosomal number determination (Mo et al. 1989) and isozymes analysis (Eastman et al. 1991). If genetic stability of somatic embryos is true, then we can consider somatic embryogenesis as a true clonal propagation method.

1.4 : MICROGRAFTING

Procedures to obtain juvenile material from mature plants are of considerable importance for the microprogation of woody plants (Ahuja 1992). Serial grafting of scions from mature trees onto juvenile rootstock often results in the temporary reappearance of juvenile characteristics (Franclet 1979). Pistachio micrografting was investigated *in vitro* as well as *in vivo* (Abousalim 1990). High levels of graft take were achieved with 10 mm (100%) and 1-3 mm (83-92%) long scions obtained from four-year-old *P. vera*. Only 33.3% micrografts survived when 0.5-0.7 mm long scions were used and no response was obtained with meristem-tips of < 0.3 mm in length.

1.5 : PRESENT SITUATION, PROBLEMS AND FUTURE PROSPECTS IN MICROPROPAGATION OF PISTACHIO TREES

In Turkey, cloning for production deployment using the *in vitro* techniques is not being attempted in pistachios. Many of the above studies are illustrated repeatedly in the literature review of chapter 1. Most orchard owners have traditionally preferred to plant or replant their orchards using seedlings resulting from sexual reproduction. Currently, the pistachio plantation is made from seedlings. Until recently, the necessary seed was usually obtained randomly rather than from the controlled crossing between clones selected for a breeding programme. The selected clones have usually been established in nursery seed orchards by grafting onto the elite rootstocks. Since pistachio is a natural outbreeder much genetic variation exists in any seed populations. Therefore, pistachio trees are grown from seed obtained as a product of open pollination and exhibit continuous variation of almost all characteristics.

Plant organ, tissue and cell culture techniques, because of the possibilities they offer for the control of the environment and nutrient conditions, are available to propagate individual trees vegetatively at a sufficient rate from clones for widespread pistachio plantations. Within the last decade, these techniques have found increasing commercial application for the rapid clonal multiplication of desirable pistachio cultivars. Up to now, there has been some success in propagation studies of pistachios carried out by means of direct organogenesis. However, as is

apparent from the above, several problems are still obstacles to use micropropagation methods as a tool for vegetative propagation of desirable pistachio trees.

A major problem is vitrification or shoot tip necrosis occurring in almost all stages of *in vitro* culture of pistachio shoots. Most of the workers who reported the occurrence of shoot tip necrosis in *in vitro* shoot cultures suspected Ca or B deficiencies as being the likely main factor involved in the development of this physiological disorder (Barghchi and Alderson 1898; Abousalim 1990). It is still unclear whether this is a physiological or chemical disorder, not only in the case of pistachio propagation but also, despite intensive study, in other species on vitrification. In the procedures developed by several workers, the approaches have been to develop a successful micropropagation technique for juvenile material and then to attempt to apply this technique to adult material. Unfortunately, until now, according to reported information, even the cultured adult pistachio trees were found to be unresponsive to *in vitro* initiation because the physiology of mature tissues differs from that of juvenile tissues and so their requirements in culture also differ (Hans Owens 1986).

One of the major problems in a wide use of micropropagation is the cost of generating plantlets *in vitro*. In general, *in vitro* multiplication has a higher unit cost than conventional propagation. Currently, the most advanced micropropagation technology available is based on the multi-staged organic process, which is extremely labour and cost intensive (Thorpe and Hassain 1988). It can be envisaged that eventually the unit cost of tissue culture propagation of economically important species will be considerably diminished through plant regeneration from somatic embryos (George and Sherrington 1984). For human consumption, the demand and trade for pistachio nuts are increasing rapidly. Moreover, population increases will ensure an adequate demand. By using micropropagation, to fully exploit the potential for intensive cropping systems, only superior proven trees should be utilised for pistachio orchards.

There should be both short- and long-term prospects for clonal propagation of pistachio trees. In the short term, using the existing methods for commercial micropropagation of pistachio trees further refinements will be necessary on the developing techniques. Also, the basic methodology of alternative techniques such as callus culture and somatic embryogenesis may be established. In the longer term the genetic improvement of this scarce plant in breeding programmes, producing commercial stocks for orchards may be considered.

The investigation described in this thesis is given under 6 Chapters:
The first chapter involves a brief introduction and an extensive literature survey.
The second chapter involves general materials and methods.

The third chapter is concerned with organogenesis of seedlings with respect to juvenility, with studies aimed at regenerating mature trees of *P.vera* L.

The fourth chapter involves embryogenesis of *P. vera* materials. More detailed studies on immature fruits, leaf explants and zygotic embryos are given. These studies are usually concerned with the initiation of embryogenic mass, maturation, germination, plantlet development and acclimatisation of SEs.

The fifth chapter involves a very brief study of the chromosome number of micopropagated *P.vera* propagules.

The general results and discussion determined in each chapter are given in the final chapter (Chapter 6) with respect to each other and to relevant literature following a brief recommendation for future *in vitro* studies.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

CHAPTER TWO : GENERAL MATERIALS AND METHODS

In this chapter, general materials and methods are described. More detailed methods and materials are given in each relevant chapter before the results presented.

2.1 : PLANT MATERIAL

Pistacia vera L. was used in all experiments reported in this thesis. All plant materials were obtained from trees of the 'Antep' cultivar of *Pistacia vera* L. (the plant materials will be referred to hereafter as *P. vera* or pistachio throughout the thesis) grown in an orchard on Ceylan-pinari state production farm, in the Urfa province of South-east Turkey. The hulls (pericarp and mesocarp) were removed and the nuts stored dry inside in plastic bags kept in the dark at 4°C. Seeds, with mean dimensions (width and length) of 12 and 20 mm respectively, were germinated, after soaking 24 h in tap water. The seeds were sown in a mixture of 1:1 sand and peat, covered with a thin layer of the soil mixture and placed in a greenhouse in Institute of Cell and Molecular Biology (ICMB). Depending on the study, explants were excised from sterilised seedlings (see sterilisation techniques, 3.1) of different age classes. In organogenesis studies, to provide a source of explants for those studies, unless otherwise acknowledged, shoots from a single seedling were bulked up by culturing shoot tip and nodal bud segment explants on MS medium supplemented with 30 gl^{-1} sucrose, 3 mgl^{-1} BAP and 0.7%. Mature plants (for meristem tip culture) were obtained from 50-year-old trees. Depending on the study, explants were excised from the same tree. The lateral branches bearing the newly-formed shoots were brought to the laboratory in plastic bags and they were stored at 4°C. In embryogenesis studies, mature and immature seeds were obtained from the same orchard. Leaf explants were obtained from aseptically germinated seedlings *in vitro* conditions. Regenerated mature leafy structures were obtained from meristem tip culture.

2.2 : PREPARATION OF CULTURE MEDIUM AND GROWTH REGULATORS

2.2.1 : Media constituents

All reagents used in this study were supplied by Sigma Chemical Co. Ltd unless otherwise acknowledged the code numbers following compound names refer to Sigma products. Unless otherwise stated, a supplemented Murashige and Skoog medium with Gamborg vitamins (M-0404) was used as the standard growth medium for organogenesis and embryogenesis studies,

except in some modification experiments where Lloyds and McCown Woody Plant medium (WP medium), Anderson's Rhododendron Basal Salt Mixture (A medium), Schenk and Hildebrandt (SH medium) media were used individually. The components are listed in Table 20. Unless otherwise stated, all media also contained 0.7% agar. Supplementation of media with carbohydrates and plant regulators for organogenesis and embryogenesis studies will be described in the appropriate sections.

2.2.2 : Preparation of media

The choice of nutritional component and growth regulator is an extremely important factor to culture the species successfully. In the account that follows all figures given are either in mgl^{-1} or percentage of w/v. In general, the basal culture medium contained MS medium minerals with Gamborg vitamins which was used at the recommended rate (4.44 gl^{-1}) in all cases except where specified. In many cases the medium was also contained 40 gl^{-1} sucrose and was solidified with 7 gl^{-1} agar, except where otherwise stated. To make up 1 litre of culture medium, first 4.44 gl^{-1} mixed powder was weighed out into a piece of foil, then transferred to a 1 litre flask and dissolved in distilled water. To avoid precipitation, a medium should always be dissolved by adding one compound at a time. Next, the sucrose was added at concentrations as specified. Afterwards, whenever other additives were required in the medium, those were added before making up the volume to 1 litre with distilled water. Prior to autoclaving the media was divided into smaller volumes (250 or 500 ml conical flasks) to which the plant growth substances (auxins and cytokinins except the-labile ones) were added, then the pH was adjusted to 5.7 or 5.8 with 0.1 M NaOH and 0.1 M HCL. If the medium was to be solidified then the required quantity of powdered agar was added and the mixture was autoclaved at a pressure of 15 PSI of 16 or 20 min depending on the volume of the medium. After autoclaving the agar media was poured into sterile tissue vessels or Petri dishes and cooled before explanting.

2.2.3 : Preparation of stock solutions of growth substances

To prepare stock solutions of growth substances the required amount of growth substance was weighed out into a piece of foil. The volumes of the various stock solutions for growth substances used in this study were changed according to the final concentrations used in the medium. After weighing, they were transferred to a 50 or 100 ml clean beaker with a small magnetic stirrer. Each compound was dissolved in a small amount (3 ml of either 1 M KOH (for TDZ, IPA, NAA, Picloram, NOA, ABA), HCl (for BAP, K, Zea, TDZ and 2iP) or absolute ethanol (for IBA, 2,4-D, 2,4,5,-T). They were stirred in distilled water until each compound dissolved and

finally made up to a volume of 50 or 100 ml distilled water. Appropriate quantities were then added to the medium prior to autoclaving (IAA, ABA, IBA if used were added after autoclaving) to achieve the desired final concentrations. Growth substance solutions were stored at 4°C and were routinely replaced with fresh solution every three weeks.

2.3 : STERILISATION TECHNIQUES

To avoid microbial contamination in the initial stage in the micropropagation process, it is essential that initiation and establishment of *P. vera* seeds, seedling explants grown in a glass-house for up to two years or mature field grown materials, as well as all nutrient media, glassware, instruments, and culture bench before use were carried out under conditions of total asepsis, because, like all plants, *P. vera* seeds and offshoots are inevitably contaminated by a wide range of fungal and bacterial micro-organisms. Therefore it is first necessary to find the best surface sterilisation of *P. vera* seeds and offshoots in order to obtain sterile explants. However, the method needed to remove all those micro-organisms should not damage the plant system in order to obtain the above objective. The sterilisation procedures followed during all *in vitro* manipulations are described below.

2.3.1 : Sterilisation by heat

The following items were sealed or wrapped in a double layer of aluminium foil and were sterilised by autoclaving at 121°C for 16 min at a steam pressure of 15 PSI.

1. Flasks containing distilled water
2. Flasks containing nutrient media lacking heat-labile compounds
3. Forceps, scalpel handle fitted with No 10 or 11 surgical blade
4. Plastic tissue culture vessels with or without medium

2.3.2 : Sterilization by filtration

The heat-labile compounds IAA, IBA, Zea, ABA, GA_3 and peroxidase were filter-sterilised using sterile Acrodisc 0.2 μm filter (Gelman Sciences 4187).

2.3.3 : Aseptic procedures used throughout

Standard sterile procedures were followed during all manipulations inside the sterile room and in the flow cabinet. 'Triflex' surgical gloves were worn which had been sterilised with absolute ethanol prior to use, at regular intervals during manipulations and immediately after handling non-sterile items. A face mask was used when working for long periods. The working surface was swabbed with absolute ethanol each time before use. The tips of forceps and scalpels were always kept in absolute ethanol and flamed each time before use.

2.3.4 : Conservation of sterility

All possible measures were taken to maintain sterility. Immediately after being taken out, the autoclaved items were transferred to the sterile room. Before placing these items in the sterile room, the surface of the bench and flasks were swabbed with absolute ethanol. Immediately after use all working surfaces were cleaned with tissues containing a few drops of Teepol solution and then with absolute ethanol.

2.3.5 : Development of sterilisation procedures for immature seed, mature seed, seedlings (grown up to two years in the greenhouse) and mature materials

Three sterilisation agents were tested: sodium hypochlorite, ethanol and hydrogen peroxide. The effectiveness of these chemicals was studied and the most suitable one or ones were selected for routine use in micropropagation of pistachio materials.

2.4 : CULTURE CONDITIONS

2.4.1 : Nutrient medium and containers

The culture conditions, including the strength of the nutrient medium, sucrose levels, culture containers, and environmental conditions, were all adjusted according to the requirements of cultures at each stage of plant regeneration. The cultures were incubated generally at 25°C in continuous photoperiod having a light intensity of 20 μmol m^{-2} s^{-1} unless otherwise acknowledged.

In all organogenesis studies, the explants were incubated on magenta GA-7 vessels containing 50 ml solidified media. For rooting treatment, induced shoots were incubated in 100 x12 mm test tubes (Sigma) containing 15 ml medium. For root induction, the treated shoots were transferred into magenta GA-7 vessels containing 50 ml of the same medium, but without growth substances and including sucrose (4%) and agar (0.7%).

2.4.2 : Hardening off and establishment of plantlets

In both organogenesis and embryogenesis studies, the regenerated plantlets were washed overnight in running water before being potted up in a sterile 1:1 mixture of peat and perlite or peat and grit. Plantlets were covered with a pyrex beaker to maintain 90 ± 5% relative humidity for at least two weeks and then the humidity was gradually reduced to 65 ± 5% before transfer into greenhouse conditions. Nutrient feed (Solinure 7, Fisons) was given to plants undergoing growth on a regular weekly basis. The regenerated plantlets via organogenesis or embryogenesis were transferred into plastic containers containing sterile horticultural substrate moistened with a 0.1% solution of N:P:K (2:1:1) (Solinure 7). The plastic containers, with 1 plantlet each, were placed for at least 4 weeks in a growth room under a 24-h photoperiod (40 $\mu mol\ m^{-2}\ s^{-1}$) with day and night temperatures of 20°C. The relative humidity in the growth room was maintained at the same level (90 ± 5%) and during the first week the plants were sprayed daily with water. After 4 or 5 weeks, the plants were placed in a greenhouse. They were potted into bigger pots with the above compost mixture.

2.4.3 : Greenhouse conditions

The greenhouse temperature was about 25°C by day; 20°C night; 18-h daylength. The greenhouse was illuminated by mercury fluorescent lamps (400 W, MBFR/U, Thorn).

2.5 : CYTOLOGICAL STUDIES

Reagents used in chromosome studies are given in Table 2. Hundreds of somatic or zygotic embryos were germinated aseptically in Petri dishes on MS medium and maintained under the same conditions. Sampling of the material: To make possible later measurements, different roots were sampled as follows:

a- first root tip up to 2mm long;

b-second root up to 2mm long and different times of the day.

To find the best conditions of germination and times of sampling the materials in order to get the most active meristems, the different combinations of the above conditions were tested.

Table 2 : Reagents used in chromosome studies. The chemicals and their method of preparation are described in Table 68; the numbers in brackets are those of this Table.

Pre-treatment	Fixation	Maceration (Hydrolysis)	Staining	Softening of tissue	Temp. mounting	Perm. mounting
1-Bromona-phtalene (1)	Farmer's (5)	5N HCl	Lacto-propionic orcein(7)	Cellulase 4% (10)	Nail Polish	Absolute alcohol
8-Hydroxy-quinolene (2)	Carnoy's (6)		Acetic acid orcein (8)	Pectinase 4% (11)	45% Acetic acid	Xylene
Colchicine (3)			Feulgen's (9)			45% Acetic acid
P-Dichloro-benzene (4)						Paraffin
						Euparal

2.6 : DATA COLLECTION

Recording of contaminated, decontaminated seeds or other surface sterilised materials were made during the culture initiation. In organogenesis studies, for assessing shoot proliferation, shoot numbers, total shoot lengths and total bud numbers were recorded generally after 4 weeks of culture. Rooting levels and numbers of roots per microshoot were recorded. In the case of embryogenesis studies, the number of seeds which produced EMS was counted after 4 weeks of culture. Dry weight and fresh weight measurements were made in the establishment of EMS suspension. The number of the somatic embryos was counted in the maturation studies from all tested treatments. The number of SEs germinated was counted after 10, 20, 30 and 40 days of culture in the agar-solidified medium, but after 14 and 21 days in the case of the liquid medium maturated embryos. The number of plantlets developed was counted after 7, 14, 21 and 28 days of culture in the agar-solidified medium.

2.7 : STATISTICAL ANALYSIS

The cultures in all experiments were set out in a completely randomised block design. Descriptive analysis was performed to provide information about the central tendency and

variability of data. To detect significant differences among treatment levels, data from factorial and non-factorial experiments were subjected to ANOVA (analysis of variance) using the Minitab package. The Student's *t*-test was adjusted at $P = 0.05$ probability level to separate mean differences when significant treatment effects were detected.

Pearson correlation was calculated between dry weight of EMSes and the concentrations of carbohydrate sources applied using the Excel 5 package.

In the case of proportional data, the Chi-square (χ^2) was used whenever appropriate. Otherwise, a logistic linear regression was performed to find out how a single dependent variable is affected by the values of one or more independent variables. A logistic regression analysis using a generalised linear model was fitted by maximum likelihood, which avoids transformation problems. A suitable linear logistic model was considered where the suitable data is constructed by writing the logit of p_{ijklm} as a linear combination of all main effects. The model was constructed starting from the basic mean response using the GENSTAT program (GENSTAT, 1988), i.e. logit (p_{ijklm}) = μ, then systematically including the explanatory variables into the model. The model was built up hierarchically such that if the importance of the two factor interaction was to be considered then the associated main effects were included in the model. Alternative models including all the main terms and interactions associated with them were fitted, but non-significant interactions were dropped out of the model. The change in residual deviance between two tested models is compared to the χ^2 distribution to determine whether there is any evidence of the added variable being a significant source of variations in the response. In order to adjust for overdispersion, the ratio of the residual deviance of the final model to the residual degrees of freedom (the residual mean square) can be obtained. This ratio should be approximately unity when the linear logistic model is thought to be correct. The developed statistical model is submitted for publication (Onay et al. 1995c).

The significance levels used were as follows:

$P > 0.05$ = non-significant

$P < 0.05$ = significant

$P < 0.01$ = highly significant

$P < 0.001$ = very highly significant.

CHAPTER THREE
ORGANOGENESIS STUDIES

CHAPTER THREE: ORGANOGENESIS STUDIES

In this part a series of experiments was carried out to develop methods for initiation, development, and multiplication of axillary shoots and nodal bud segments using explants from seedlings and mature trees. Here, the results are generally presented within four sections according the stages of plant regeneration.

Section 3.1 comprises investigations into the establishment of axenic cultures from seed or immature seed, explants excised from greenhouse-grown seedlings and mature trees. The objective of these experiments was to obtain an axenic culture material for tissue culture studies. Accordingly, the screening experiments were aimed to determine the effects of sterilant agents such as ethanol, NaOCl and H_2O_2, as well as testing different concentrations and time combinations of the sterilant agents.

Section 3.2 comprises investigations into the formation of axillary shoots or buds which develop on cultured nodal bud segments which have been cloned from a greenhouse-grown seedlings. The objective of these experiments was to formulate a protocol of basic cultural conditions for the optimum production of axillary shoots on these juvenile explants. Correspondingly, screening experiments were aimed to determine the effects of factors such as type and concentration of growth regulators, light, media and temperature etc.. The developmental potential of explants, using optimum culture conditions formulated as described above, is examined in terms of influence of age of the donor seedlings. Methods are described which do in fact, achieve high rates of multiplication in terms of shoot number and bud number, and which may form the basis of a practical scheme for further use.

In section 3.3, attempts are described to initiate an establishment of meristem tip culture from 50-year-old trees. The role of the explant, the time of the year that the explant was harvested, the type of explant used, the role of exogenous factors, such as applied cytokinins on the establishment of shoot initiation were examined.

In section 3.4, the experiments were directed towards the development of a sequential treatment, mainly using exogenous growth substances, to produce uniform propagules, developed from cultured axillary buds. The aim was to produce plantlets in which the root system would be highly branched, and with balanced growth between root and shoot. Once a suitable protocol had been established, it was applied to the rooting of shoots produced on seedlings and those induced on explanted axillary shoots.

3.1 : DEVELOPMENT OF STERILISATION PROCEDURES FOR PISTACHIO MATURE SEEDS OR IMMATURE SEEDS AND SEEDLING MATERIALS GROWN UP TO TWO YEARS IN THE GREENHOUSE AND MATURE MERISTEM TIPS (50-YEAR-OLD)

In this section, a series of experiments were conducted to investigate the effects of various sterilant agents involved in the process of obtaining axenic tissue culture materials from mature seed or immature seeds, seedlings and meristem tip explants of pistachio. Three sterilisation agents were used, sodium hypochlorite, ethanol and hydrogen peroxide. The effectiveness of these chemicals was studied and the most suitable one was selected for routine use in micropropagation of pistachio trees. Surface sterilisation in all the subsequent experiments was carried out using a shaker.

3.1.1 : A preliminary study on germination capacity of unsterilised pistachio seeds

The aim of this experiment was to obtain preliminary data to determine whether or not seeds of pistachio need a sterilisation treatment. Therefore in this experiment, in the first replication of 20 untreated seeds with which the extracted kernels were germinated on thin towels in a sterile plastic container, Sigma Phytotray, moistened at 25°C under a continuous photoperiod provided by fluorescent tubes. Observations were made after seven days of incubation and again after 14 days.

Under the standard germination test it was observed that 60% of the seeds were visibly contaminated with fungi and bacteria in seven days. After 14 days of incubation, 90% of the seeds were contaminated, nevertheless two seeds had germinated, with vigorous shoots and radicles. The results provide clear evidence that pistachio seeds need a sterilisation treatment before attempting germination in *vitro* conditions. The following experiment was performed in order to investigate the treatment necessary to achieve sterilisation.

3.1.2 : Experiment to investigate if sodium hypochlorite sterilising solutions affect decontamination and germination of pistachio seeds

In the previous experiment, a high level of contamination was recorded after seeds were incubated without any sterilisation treatment, therefore in this preliminary study, 10%, 15% and 20% hypochlorite together with an untreated control were used to identify the effect of sterilisation on the decontamination and axenic germination of pistachio seeds.

The first replications of 16 kernels, from which the shell had been removed were immersed in the 5%, 10% and 20% NaOCl (v/v) x 10 min treatments, together with a no treatment control,

then washed in sterile distilled water three times and placed on germination deeply embossed thin paper towels in a sterile plastic plant tissue vessel with approximately 10 ml of distilled water. The observations were taken after four days, eight days and again after 14 days.

The frequencies of germinated and contaminated seeds varied significantly with the different concentrations of sodium hypochlorite (Table 3, $P < 0.05$). At the lower level of concentration of NaOCl (5% and 10%), high levels of contamination (75%) were observed after 14 days of incubation. The results presented in Table 3 show clearly that the increasing NaOCl concentration reduced the frequency of the contaminated pistachio seeds that were incubated for 14 days and that the surface sterilisation techniques using sodium hypochlorite at various concentrations had an effect on the decontamination of the seeds. The results also indicate that the pistachio seeds may not be decontaminated in the 20% of hypochlorite because the frequency of germinated seeds (62.5%) is not a suitable level for future tissue culture techniques. The 20% sodium hypochlorite x 10 min treatment gave the best results, with a germination level of 62.5%, while the lowest one with the nil treatment, 12.5%. In other words, increased concentration resulted in a lower level of contamination.

Table 3 : The effect of NaOCl on decontamination and germination of pistachio seeds.

Treatments	% of seeds germinated (not malformed)	% of seeds contaminated
Nil	12.5	87.5
5% NaOCl x 10 min	25.0	75.0
10% NaOCl x 10 min	25.0	75.0
20% NaOCl x 10 min	62.5	37.5
χ^2 test (3 df)	$P < 0.05$	$P < 0.05$

Figures presented are the means of 16 replicates observed on day 14 of culture.

Germination of seeds. Initially the seed produces a radicle within 40 h after incubating. A coleoptile, probably a much modified first leaf which grows, enclosing the epicotyl in the embryo of seeds, usually appears 24 h after the culture. Root formation and growth are usually higher than shoot. Stem elongation often occurs within seven days of vegetative growth occurring between the primary and secondary leaves. The depth of the pistachio root system usually grew to 50 cm in Petri dishes within 14 days.

3.1.3 : Effect of optimum immersion time in NaOCl on the decontamination and germination of pistachio seeds

As presented in the previous experiment, the 20% sodium hypochlorite treatment was selected as the optimum combination of concentration in NaOCl for the decontamination and germination

of pistachio seeds. This experiment was performed to obtain the optimum immersion time of NaOCl used in the 20% NaOCl solution. The experimental procedure followed was as with the previous experiment. The results obtained are shown in Table 4.

There was a significant difference in the frequencies of germinated and contaminated seeds between those that had undergone treatments ($P < 0.01$, Table 4). As can be seen from Table 4 there is an obvious pattern in the frequencies governed by the immersion times for seed decontamination. The frequency of the decontaminated seeds increases with an increase in the immersion times and reaches the highest frequency, 68.75% with the 20% NaOCl x 20 min treatment after 14 days.

Table 4 : The effect of NaOCl solution in different immersion times on decontamination and germination of pistachio seeds.

Treatments	% of seeds germinated (not malformed)	% of seeds contaminated
Nil	0	100
5 min in %20 NaOCl	37.5	62.5
10 min in %20 NaOCl	31.2	68.7
15 min in 20% NaOCl	50.0	50.0
20 min in 20% NaOCl	68.7	31.2
χ^2 test (4 df)	$P < 0.01$	$P < 0.01$

Figures presented are the means of 16 replicates observed on day 14 of culture.

The results presented in Table 4 lead to the conclusion that 20% NaOCl x 20 min treatment may be the optimum immersion time in NaOCl to sterilise pistachio seeds because there were no oversterilized or malformed seeds, even after the highest immersion time. Surface sterilising techniques using the 20% sodium hypochlorite at various immersion times had effect on the decontamination of pistachio seeds. However, the results presented in Table 4 show no clear combination that provides a level of contamination suitable (10%) for future tissue culture techniques. I have therefore established the subsequent experiment in order to find the most suitable surface sterilisation of pistachio seeds.

3.1.4 : A study of the effect of sodium hypochlorite solution and higher immersion times on the decontamination of pistachio seeds

The technique described above was not completely effective at removing contaminants on cultures of pistachio seeds in order to obtain the sterile seedling for the culture initiation and establishment. Therefore this experiment was undertaken to overcome the problem of contamination of the cultured explants.

In this experiment, one solution of NaOCl (20%) was used in combination with four immersion times together with a nil treatment (20, 30, 60 and 90 minutes). In the first treatment, the first replication of 32 kernels, from which the shell had been removed, were presterilized in absolute ethanol, for 2 min followed by 20% (v/v) hypochlorite for 20 min, rinsed three times in sterile distilled water, and then 16 kernels were placed on a paper towel in a plastic plant tissue culture vessel together with 10 ml of distilled water. The other 16 seeds were divided transversely into several pieces and then immediately cultured in 9 cm Petri dishes containing 20 ml of sterile Potato Dextrose Agar (a medium for culturing bacteria, yeast and moulds). This was repeated with all 16 seeds. Finally each Petri dish was sealed with parafilm and incubated in the culture room at 25°C. The final observation of the cultures was recorded after one week and again after an incubation period of two weeks.

Table 5a : The effect of NaOCl solution in higher immersion times on the decontamination of the chopped pistachio seeds.

Treatments	% of seeds germinated (not malformed)	% of seeds contaminated	% of seeds malformed
Nil	0	100	0
20 min in 20% NaOCl	62.5	25.0	12.5
30 min in 20% NaOCl	50.0	25.0	25.0
60 min in 20% NaOCl	43.7	12.5	43.7
90 min in 20% NaOCl	25.0	12.5	62.5
χ^2 test (4 df)	P < 0.01	P < 0.01	P < 0.01

Figures presented are the means of 16 replicates observed on day 14 of culture.

The frequencies of % of seeds germinated, % of seeds contaminated and % of seeds malformed are given in Table 5a and b. The frequencies of germinated, contaminated and malformed kernels varied significantly with different immersion times together with the 20% NaOCl in both types of incubation (P < 0.01, P < 0.01, P < 0.01, Table 5a, and P < 0.05, P < 0.01 and P < 0.01, Table 5b, respectively). In both types of incubation, the malformed kernels' rates varied from 12.5% to 62.5%. The prolongation of the immersion time from 20 min to 90 min in 20% NaOCl had a positive effect on the number of seeds malformed in both types of incubation. All treatments showed contamination with at least a level of 12.5% in the 90 min in 20% NaOCl and a level of 12.5% in the 60 min through the chopped kernels and intact kernels respectively. Generally, however, as the immersion time increased the percentage of contamination decreased, but malformation increased (Table 5a and b). The highest frequency of axenic germination occurred with the chopped seeds, 62.5%. Within 14 days of explanting some cultures were contaminated with only fungal contaminants and soon died. The results presented in this experiment again showed that the surface sterilisation technique using 20% NaOCl at various periods was not completely effective on the axenic germination of pistachio

seeds for the use of further tissue culture studies. Therefore, decontamination of pistachio seeds needs an alternative sterilisation technique.

Table 5b : The effect of NaOCl solution in higher immersion times on the decontamination of the intact pistachio seeds.

Treatments	% of seeds germinated (not malformed)	% of seeds contaminated	% of seeds malformed
Nil	0	10	0
20 min in 20% NaOCl	56.2	18.7	25.0
30 min in 20% NaOCl	43.7	12.5	43.7
60 min in 20% NaOCl	37.5	12.5	50.0
90 min in 20% NaOCl	37.5	0	62.5
χ^2 test (4 df)	$P < 0.05$	$P < 0.01$	$P < 0.01$

Figures presented are the means of 16 replicates observed on day 14 of culture.

3.1.5 : Determination of the source of fungal or bacterial contaminants on the pistachio seeds

Seeds were presterilized by immersing in absolute ethanol for 2 min followed by sodium hypochlorite solutions (20%, 50% and 100%) for 20 min. They were then washed in sterile distilled water and blotted dry. Next, the kernels were chopped transversely and cultured in 9 cm in the Petri dishes containing 20 ml of Potato Dextrose Agar as explained in the previous experiment. There were 16 replicates for each treatment.

Within 24 h, contamination was observed on some plates throughout the treatments. As can be seen from Table 6, there was a significant difference in the frequencies of germinated, contaminated and malformed seeds between those that had undergone treatments ($P < 0.01$). After 14 days of incubation a level of 18.75% contamination was reached at 100% NaOCl but gave the germination level of 56.25% of normal axenic seedlings. The quality of germinated seeds was affected by the immersion time after 14 days of culture, and germination rates varied from 6.25% to 68.75% (Table 6). The considerable contamination with 18.75% in 100% NaOCl of the chopped seeds suggests that the source of contamination comes from within the seeds. As the results described in this experiment again showed contamination of pistachio seeds, using commercial sodium hypocholorite alone at various concentrations and periods (Table 3, 4, 5 and 6) had no effect on the decontamination of pistachio seeds in order to obtain axenic seedlings for further studies in plant tissue culture techniques. An alternative sterilisation technique using hydrogen peroxide was tested in the following experiments.

Table 6 : The effect of NaOCl solution in higher concentrations of hypochlorite on the decontamination and axenic germination of pistachio seeds.

Treatments	% of seeds germinated (not malformed)	% of seeds contaminated	% of seeds malformed
Nil	6.25	93.7	0
20 min in 20% NaOCl	68.7	31.2	12.5
20 min in 50% NaOCl	43.7	31.2	25.0
20 min in 100% NaOCl	56.2	18.7	25.0
χ^2 test (3 df)	P < 0.01	P < 0.01	P < 0.01

Figures presented are the means of 16 replicates observed on day 14 of culture.

3.1.6 : The effect of H_2O_2 on the decontamination of pistachio seeds

In this experiment, H_2O_2 was used as a sterilant agent to overcome the problems of contamination caused by endogenous or exogenous bacteria and fungi.

Table 7 : The effect of H_2O_2 on the decontamination of the pistachio seeds.

Treatments	% of seeds germinated (not malformed)	% of seeds contaminated
Nil	0	100
10 min in 5% H_2O_2	20	80
10 min in 10% H_2O_2	40	60
10 min in 15% H_2O_2	30	70
χ^2 test (3 df)	P < 0.05	P < 0.05

Figures presented are the means of 10 replicates observed on day 14 of culture.

A preliminary test was carried out using 5%, 10% and 15% H_2O_2 (w/v) solution prepared immediately before use for 10 min with pistachio seeds. The replications of 10 kernels, from which the shell had been removed, were presterilized in absolute ethanol for 1 min followed by 5%, 10% and 15% (w/v) hydrogen peroxide solutions for 10 min, then rinsed three times in sterile distilled water. The kernels were divided transversely into several pieces and then immediately cultured in 9 mm Petri dishes containing 20 ml of potato dextrose agar. The experiment was done once and the average results of this experiment are given in Table 7. As can be seen from the results presented in Table 7, it was clear that considerable contamination was observed using concentrations up to 15% hydrogen peroxide. Chi-square analysis of the data showed that there was evidence of a significant difference in the frequencies of germinated and contaminated kernels between those that had undergone treatments ($P < 0.05$).

After 14 days of culture, only 40% of the 10 min in 10% H_2O_2 treatment had germinated kernel while 60% had contaminated with fungi and bacteria. Therefore, as higher contamination was observed in the preliminary test, further experiments with hydrogen peroxide were not carried out.

3.1.7 : The effect of three sterilising agents for axenic germination of the pistachio seeds

Erratic results were obtained by using sodium hypochlorite and hydrogen peroxide in the previous experiments. In similar cases, more than half of the seeds germinated, but were contaminated, with the establishment of shoot cultures or the initiation of callus cultures. These results show clearly that an effective sterilisation method for the production of sterile seedlings for the future tissue culture techniques from pistachio seeds was not achieved up to now in the studies. Therefore I decided to use three sterilising agents (ethanol, sodium hypocholorite and hydrogen peroxide) for the axenic germination of the pistachio seeds.

Table 8 : The effect of three surface sterilising agents for axenic germination of the pistachio seeds.

Treatments	% of seeds germinated (not malformed)	% of seeds contaminated	% of seeds malformed
Nil	0	100	0
2 min in absolute ethanol 10 min in 10% H_2O_2 20 min in 20% NaOCl	93.7	6.2	0
2 min in absolute ethanol 10 min in 10% H_2O_2 30 min in 20% NaOCl	87.5	0	12.5
χ^2 test (2 df)	$P < 0.01$	$P < 0.01$	$P < 0.01$

Figures presented are the means of 16 replicates observed on day 14 of culture.

In this experiment, first 32 kernels from which shell had been removed were presterilised in absolute ethanol for 2 min. Next, the presterilised kernels were sterilised in 10% H_2O_2 for 10 min followed by 20% (v/v) NaOCl for 20 min and 30 min, rinsed three times in sterile distilled water, and then 16 kernels with testa and the rest without testa were incubated on SH medium without any growth regulator. There was a higher level of malformed kernels cultured with testa in this experiment. Therefore, only the results of the kernels cultured without testa using the three sterilising solutions are shown in Table 8. Chi-square analysis of data showed that there was evidence of a significant difference in the frequencies of germinated, contaminated and

malformed kernels between those that had undergone treatments ($P < 0.01$). In the 30 min in 20% NaOCl treatment none of the kernels was contaminated but 12.5% of the seeds were oversterilised or malformed. However, the results obtained from the 20 min in 20% NaOCl treatment show a clear combination that provides a level of contamination suitable (6.25%) for future tissue culture techniques. No abnormalities of the kernels without testa were observed for the 20 min in 20% hypochlorite treatment observed after 14 days of incubation. As a result of this study, the sterilising solutions of absolute ethanol for 2 min, 10% H_2O_2 for 10 min and 20% NaOCl for 20 min were chosen for future sterilisation of pistachio seeds.

3.1.8 : The effect of pre-sterilisation on decontamination of pistachio seeds

It has been established that successful decontamination of pistachio seeds by hydrogen peroxide and sodium hypochlorite is possible in association with pre-sterilisation. It was then tested whether or not presterilisation was required for this decontamination procedure. Therefore, the successful treatment in the previous experiment was repeated with and without a pre-treatment. The details of the treatments and results obtained are shown in Table 9.

Table 9 : The effect of pre-sterilisation on decontamination of pistachio seeds.

Treatments	% of seeds contaminated	% of seeds decontaminated
2 min absolute ethanol 10 min in 10% H_2O_2 20 min in 20% NaOCl	6.25	93.7
10 min in 10% H_2O_2 20 min in 20% NaOCl	12.5	87.5
χ^2 test (1 df)	N.S.*	N.S.*

**Indicates that there is no significant difference at $P = 0.05$ level of significance. Each figure represents the mean of two experiments with 16 replicates per treatment in each experiment, observed on day 14 of culture.*

There was no evidence of a significant difference in the frequencies of germination and contaminated seeds between those that had undergone treatments, although the treatment without presterilisation gave higher frequency of the contaminated seeds (12.5%, Table 9). It appeared from these results that pre-sterilisation may not be required for mature pistachio seeds. The surface sterilisation method developed to mature seeds was applied for immature seeds and 100% success was obtained. As a brief presterilisation (2 min) with absolute ethanol was not found to be detrimental to the germination of pistachio seeds in culture, all seeds were

similarly pre-sterilised for convenience. Absolute ethanol may not be used for pre-sterilisation of pistachio immature seeds.

3.1.9 : A preliminary study of the effect of the surface sterilising method developed for seeds on the decontamination and growth of pistachio apical tips and nodal bud segments grown under greenhouse conditions

The aim of this experiment was to obtain preliminary data in order to decide whether or not the surface sterilisation method developed for pistachio seeds could be used to sterilise the pistachio seedlings grown in the greenhouse for up to two years. Freshly harvested apical and nodal bud segments were sealed with wax on the cut edges to maintain availability for multiple shoot initiation. After presterilization for 2 min in absolute ethanol, the explants were treated for 10 min with H_2O_2 and 20 min with 20% hypochlorite. After washing thoroughly with sterile distilled water, explant were prepared after removal of the waxed parts and the excess tissue. Then, the nodal bud segments and apical tips were cut into pieces, 10-12 mm in length, and cultured for 4 weeks as detailed in the section 3.2 for multiplication of shoots.

The results presented in Table 10 show that the various ages of donor seedling were affected differently by the surface sterilisation method used. There were significant differences in the frequencies of uncontaminated and oversterilised explants between the results obtained from the different ages of donor seedlings ($P < 0.01$). All of the one-month-old seedling explants were oversterilised, but none of the other tested treatments showed any oversterilisation.

Table 10 : The efficiency of the surface sterilisation method developed for seeds on the decontamination and growth of pistachio apical and nodal bud segments grown in the greenhouse*.

Age of donor seedling (month)	% of explant uncontaminated	% of explant contaminated	% of explant oversterilised
1	0	0	100
3	100	0	0
9	100	0	0
15	83.3	16.7	0
χ^2 test (3 df)	$P < 0.01$	N.S.**	$P < 0.01$

**Figures presented are the means of 12 replicates observed on day 30 of culture. **indicates that there is no significant difference at $P = 0.05$ level of significance.*

Contamination was only recorded in the 15-month-old seedlings. There was no evidence of a significant difference in the frequencies of the contaminated explants between the treatments. Growth of explants showed no difference between the treatments. The results also indicate that

tissue from mature plants may be decontaminated by this method. The following experiment was carried out to investigate this.

3.1.10 : A preliminary study of the effect of the surface sterilising method developed for seedlings on the decontamination and growth of pistachio apical and nodal bud segments from field-grown-mature trees

In the present experiment, the procedure for sterilising pistachio seeds and seedling materials was investigated for its capacity to sterilise mature materials. Apical tips and nodal bud segments were collected from actively growing shoots of 50-year-old pistachio trees growing on the state production farm of South-east Turkey. The explants were shipped to Edinburgh by plane within three days. The explants were surface-sterilised by a 2-min dip in absolute ethanol followed by a 10-min sterilisation in 10% H_2O_2 and a 20-min soak in 20% NaOCl. After 3 rinses in sterile water, the explants were cultured in the two ways detailed in section 3.3.1 in magenta GA-7 vessels containing agar-solidified MS medium supplemented with 3% sucrose and 1 mgl^{-1} BAP:

1. After removal of the waxed cut ends, the whole buds were cultured.
2. Meristem tips were excised from the sterile explants and incubated for 30 days.

Percentages of explants which were contaminated, uncontaminated or browned are summarised in Table 11. There was a significant difference in the frequencies of contaminated, uncontaminated and browned explants between the results obtained from buds and meristem tips ($P < 0.01$). The percentage of the explants contaminated for buds (50%) after 30 days of culture was higher than for meristem tip, which showed an acceptable rate of contamination (10%).

Table 11 : The effect of the use of the surface sterilisation method developed for seeds and seedlings on decontamination and growth of mature field-grown *P. vera* material.

Type of explants cultured	% of explants contaminated	% of explants uncontaminated	% of medium browning
Buds (10-12 mm)	50	50	100
Meristem tips (less than 1 mm)	10	90	50
χ^2 test (1 df)	$P < 0.01$	$P < 0.01$	$P < 0.01$

Figures presented are the means of 20 replicates observed on day 30 of culture.

The results presented in Table 11 lead to the conclusion that incubation of meristem tip cultures for 4 weeks resulted in a significant reduction in media browning, as compared with culturing

them using buds. Medium browning was always very high when a large bud was used. It was observed that smaller explants induced lesser browning. None of the cultured bud explants regenerated or proliferated any tissue during the period of the experiment. Contamination of explants increased significantly when buds were used as the source of the initiation material. None of the cultured explants produced callusing tissues. No fungal contamination was observed in either cases and all the infection observed was of bacterial origins. Therefore, meristem tips were used in order to initiate cultures from mature materials.

3.1.11 : Discussion

An effective surface sterilisation method for the production of sterile explants from *P. vera* mature seeds or immature kernels, seedlings, and mature meristem tips was achieved. Eradication of fungal and bacterial contaminants has been encountered as a major problem in the establishment of pistachio tissue culture by many other workers (Barghchi 1982; Bustamante-Garcia 1984 and Abousalim 1990). None of these authors systematically searched for the optimum procedure, although Abousalim (1990) gave optimum conditions for certain materials. Consequently, a thorough comparison was made of different surface-sterilising agents, used for different periods of time on the explants of *P. vera*. Initial results (i.e. 3.1.2 and 3.1.3) are in agreement with those reported by Barghchi (1982) in that fungal or bacterial contaminants contained within the otherwise surface sterilised seeds produced high levels of contamination. However, data on the effectiveness of the sterilisation method have not been published by other workers except by Abousalim (1990). Results obtained here are not in agreement with those reported by Abousalim (1990) using 20% NaOCl as a sterilant. Surface sterilisation of pistachio seeds was achieved by Abousalim (1990) using only 20% NaOCl solution, supplemented with 0.1% Tween 20 as a wetting agent, using a shaker or a vacuum infiltration system. In the case of shaker treated seeds, 13.5 and 2.7% fungal contamination was observed when 12 and 20% NaOCl were used respectively (Abousalim 1990). Fungal contamination levels which produced the present results (less than 10%) after 14 days incubation in similar conditions are comparable to Abousalim's results. Parfitt and Almendi (1994) controlled contamination through the use of a 2% CO_2 atmosphere, increased light levels to promote photosynthesis, and elimination of all carbon sources from the substrate. In the present work erratic results were obtained (Table 7) when H_2O_2 was used as a sterilant. As higher contamination was observed in the preliminary results, further experiments with H_2O_2 were not carried out. Axenic germination of pistachio seeds in sterile conditions may be achieved using three sterilising agents (ethanol, sodium hypochlorite and hydrogen peroxide). However, it appears from the results shown in Table 9 that presterilisation was not required for pistachio seeds whereas it was essential for the seedlings and mature plants. Vigorous sterilisation procedures of mature pistachio seeds are not recommended as tissues proved

sensitive to longer periods in higher concentrations of NaOCl (Table 5a and b) producing malformed seedlings. My results showed that for periods equal or longer than 60 minutes in 20% NaOCl a higher percentage (43.75%) of malformed seedling were observed.

In the case of explants from adult pistachio materials, decontamination was best achieved when explants were obtained from actively-growing meristem tip cultures of 50-year-old *P. vera* L. As previously noted (section 3.1.10), when nodal bud segments and meristem tips were used for the initiation of culture, a high level of media browning was observed. No benefit was gained from the use of any of the antibrowning compounds tested. However, by culturing the meristem tips, up to 80% survival was obtained using explants derived from 50-year-old trees. Axillary shoot proliferation was not observed because of increases in necrosis when the surviving explants are recultured or subcultured. Difficulties in securing a long term supply of adult materials prevent a more detailed investigation into the establishment of such mature materials. These tests, conducted with all surface-sterilising agents mentioned here, are the first such systematic study. Consequently surface sterilisation of *P.vera* mature seeds, immature fruits, seedlings up to two years-old and mature meristem tips may be achieved in ethanol (100%) for 2 min, 10% H_2O_2 for 10 min and 20% NaOCl for 20 min.

3.2 : AXILLARY SHOOT FORMATION ON CULTURED SEEDLING NODAL BUD SEGMENTS

Seeds resulting from open pollinated trees were collected from 30-year-old Antep trees grown at the State Production Farm in Urfa province of South-east Turkey. Seeds were germinated in a soil mix in greenhouse. After one year, nodal bud segments of the one of best growing seedlings were harvested, and surface-sterilised as explained in section 3.1.9. After the surface sterilisation, the basal end of the stem tissue was trimmed (generally 10-12 mm explant length) and cultured. The medium was Murashige and Skoog's medium (Sigma, M 0404) with 3 mgl^{-1} BAP as the sole plant growth regulator. Every 3 or 4 weeks, the regenerated axillary shoots were recultured or subcultured. The medium was dispensed into magenta GA-7 vessels (4 or 5 nodal bud segments per vessel) containing 50 ml aliquots of the above media. The pH of the medium was adjusted with 0.1 N KOH and 0.1 HCL before adding agar, followed by autoclaving for 15 min at 121°C. All cultures were placed in a growth room at 25°C with a 24-h photoperiod (cool-white fluorescent tubes at 20 $\mu m\ s^{-1}\ m^{-2}$). Unless otherwise stated, shoots were excised into 10-12 mm long explants each containing one nodal bud segment and cultured on agar-solidified medium. The culture vessels were covered with parafilm and incubated in a growth room at 25°C/continued light unless otherwise stated. Unless otherwise acknowledged, all the subsequent experiments were done using this clonal stock. Unless otherwise noted, all the subsequent experiments were done using the previous optimal conditions. A series of

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experiments was conducted to investigate the effects of various factors involved in the process of shoot regeneration. The number of the cultures per treatment has been given in the results in each experiment. Shoot number, shoot length and bud number data were analysed by analyses of variance. The Student's *t*-test was adjusted at $p = 0.05$ probability level to separate mean differences when significant treatment effects were detected. Data on the occurrence of necrosis and callus production were analysis using χ^2 to test for significance means. The values are expressed as percentages in the table for ease of comparison.

3.2.1 : Development of culture conditions for *in vitro* culture initiation and multiplication in the pistachio shoot cultures

The culture conditions necessary to promote successful culture initiation were investigated using twice-subcultured apical tips and nodal bud segments of *P. vera*.

3.2.1.1 : The effect of pH of the medium on the development of *P. vera* explants in culture

In this experiment the influence of pH upon shoot number and shoot length was investigated to determine the optimal level for future use. The axenic nodal bud segments were placed on MS medium supplemented with 3 mgl^{-1} BAP Gamborg vitamins plus 3% sucrose and 0.7% agar. Medium was compared for different pH: 5.6, 5.7, 5.8, 6.0, 6.5, and 7. All cultures were incubated under conditions as described in section 3.2. The average shoot length and the average number of the shoots produced were assessed after 30 days of culture.

Table 12 : The effect of the initial pH of the medium on average shoot length and average shoot number in the shoot culture of *P. vera* on day 30 of culture.

Initial pH	Average shoot number ± SE	Average shoot length (mm) ± SE
5.6	2.8 ± 0.2 a	23.9 ± 0.5 a
5.7	3.2 ± 0.2 a	28.8 ± 1.0 b
5.8	3.5 ± 0.3 a	27.0 ± 1.2 b
6.0	3.0 ± 0.2 a	25.4 ± 1.1 ab
6.5	2.8 ± 0.3 a	20.6 ± 1.0 c
7.0	2.0 ± 0.2 b	17.4 ± 1.0 d

Results presented are the means of 20 replicates with the standard errors of the means. Means in a column above followed by the same lowercase letter are not statistically different at P = 0.05 level of significance according to the Student's t-test.

Shoot bud development was clearly visible on explants after three to five days on MS medium. All explants produced shoots but differed with respect to the mean number of shoots per explant

and the mean shoot length per explant. Multiple shoots were obtained in all treatments tested but these shoots produced in the pH 6.5 and 7.0 treatments were poor in terms of vigour, colour and ease of handling. At the end of the incubation period the mean shoot length at medium pH 5.7 and 5.8 was significantly greater than that at pH 6.5 and 7 at the 5% significance level (Table 12). The 6.5 and 7.0 treatments produced the lowest mean shoot length. The mean shoot length of the lowest level pH treatment (pH 5.6) is also significantly different from the pH 5.7 treatment. The highest number of shoots was obtained at pH 5.8. The mean shoot number of the various pH treatments were not significantly different from each other. However, the medium with an initial pH of 7.0 produced the lowest mean shoot number.

In general, the pH 5.7, 5.8 treatments showed better growth and elongating shoots acquired normal appearances in external form. In conclusion it may be suggested that an initial pH in the culture medium of between 5.6 and 6.0 is optimal for the production of a higher number of regenerated shoots of the pistachio seedlings *in vitro* in terms of the mean shoot length and mean shoot number obtained in this experiment. In the following experiment the effect of agar concentration was studied.

3.2.1.2 : Modification of agar supply

In this experiment the influence of agar (Sigma A1296) concentration upon culture development was investigated to determine the optimal concentration for future use. Five concentrations of agar (Agar-Agar; Gum Agar) were prepared 0.5, 0.6, 0.7, 0.75 and 0.8%. The axenic nodal bud segments were placed on MS medium supplemented with 3 mgl^{-1} BAP Gamborgh vitamins plus 2% sucrose as described in the previous experiment. The pH of the medium was adjusted to 5.7 before adding agar. All cultures were incubated under conditions as described in section 3.2. Mean shoot number and length were recorded after 30 days in culture.

Table 13 : The effect of agar concentration on mean shoot length and mean shoot number in the shoot culture of *P. vera* on day 30 of culture.

Agar concentration (%)	Average shoot number ± SE	Average shoot length (mm) ± SE
0.50	2.0 ± 0.1 a	18.1 ± 0.7 a
0.60	2.7 ± 0.2 ab	18.9 ± 0.7 a
0.70	3.2 ± 0.3 b	18.0 ± 0.5 a
0.75	2.2 ± 0.2 a	17.0 ± 0.8 ab
0.80	1.8 ± 0.1 a	15.8 ± 0.7 b

Results presented are the means of 20 replicates with the standard errors of the means. Means in a column above followed by the same lowercase letter are not statistically different at P = 0.05 level of significance according to the Student's t-test.

On MS medium, significant differences were observed in both mean shoot number and mean shoot length between the tested agar concentrations (Table 13). Explants on 0.7% agar produced the most shoots. Although more than two shoots were produced per explant on 0.5% and 0.75% agar treatments, the multiplication rate was significantly lower than in the 0.7% agar treatment. Significantly higher rates of shoot elongation were obtained with lower concentrations of agar (0.5%, 0.6% and 0.7%) than at the highest, 0.8%. Callus was produced at the base of shoots when the cultures were maintained for more than four weeks on any treatment tested. The general appearance and growth habits of shoots seems to be better on medium with the 0.7% treatment. Therefore, agar concentration 0.7% was chosen for further studies.

3.2.1.3 : Modification of sucrose supply

In order to optimise the sucrose concentration in the medium, three concentrations (1%, 2% and 3% w/v together with a control medium without sucrose, were tested with MS as the basal medium. The axenic axillary shoots (subcultured twice) were placed on MS medium supplemented with 3 mgl^{-1} BAP Gamborg vitamins plus 0.7% agar. All cultures were incubated under conditions as described in section 3.2. Mean shoot number and length were recorded after 30 days in culture.

Table 14 : The effect of sucrose concentration of the medium on mean shoot length and mean shoot number in the shoot culture of *P. vera* on day 30 of culture.

Sucrose conc. (%)	Average shoot number ± SE	Average shoot length (mm) ± SE
0.0	1.0 ± 0.0 a	13.4 ± 0.2 a
1.0	2.0 ± 0.1 b	23.3 ± 0.6 b
2.0	2.9 ± 0.1 c	27.1 ± 0.8 c
3.0	3.1 ± 0.2 c	30.4 ± 0.8 d

Results presented are the means of 20 replicates with the standard errors of the means. Means in a column if followed by the same lowercase letter are not statistically different at P = 0.05 level of significance according to the Student's t-test.

Explants on 3% sucrose produced the largest number and longest shoots. However, the means were not significantly greater than for the 2% level, although they were significantly greater than for the 1% and control treatments (Table 14). The results in Table 14 show that shoot number and length increased with increasing sucrose concentration in the range tested. Generally, proliferated shoots appeared healthy and grew well. However, none of the explants in the control treatments showed any sign of proliferation. The results clearly show that sucrose influenced shoot multiplication in the nodal bud segments of *P. vera* seedlings. Sucrose at the 3% level was

optimal in promoting axillary shoot formation and in the absence of sucrose no proliferation was observed at the conclusion of the study. Thus, the 3% sucrose may be used as a minimal level in promoting axillary shoot formation.

3.2.2 : Influence of growth substances on shoot growth and number

It was evident that the cultured axillary bud and nodal bud segments exhibited different quantitative organogenic responses according to the nature of the growth substance applied (Bustamenta-Garcia 1984, Barghchi 1982 and Abousalim 1990).

3.2.2.1 : Influence of cytokinins on the shoot number, shoot length and bud number of cultured explants from seedlings of *P. vera*

Using MS medium as the control, the effect of zero cytokinins as well as of BAP, Kin, 2iP and Zeatin each at 3 mgl^{-1} were examined. Nodal bud segments (subcultured 3 times) containing 3 nodes were used. All cultures were incubated under conditions as described in section 3.2. The mean number of the shoot length and the mean number of the shoots, the mean number of the buds, the mean number of the leaves per explant and the percentage of shoots showing apical necrosis were measured after 30 days of culture.

Table 15 : Effects of different cytokinins on the shoot number, shoot length, bud number and leaf number of cultured explants from seedlings of *P. vera* after 30 days on media containing cytokinins*.

	Control	BAP	K	2iP	Zea	F test (49 df)	χ^2 test (4 df)
Mean shoot number	1.0 a (0.0)	4.6 b (1.1)	3.3 b (1.0)	1.3 c (0.4)	1.6 c (0.5)	P < 0.01	
Mean shoot length (mm)	14.5 a (1.1)	30.4 b (2.1)	25.2 b (2.6)	23.3 b (3.3)	26.3 b (3.2)	P < 0.01	
Mean bud number	3.7 a (0.2)	23.2 b (4.6)	18.0 b (4.0)	5.2 a (0.8	8.6 c (1.5)	P < 0.001	
Mean leaf number	2.7a (0.2)	18.7 b (3.7)	14.8 b (3.2)	4.0 c (0.8)	7.1 d (1.3)	P < 0.001	
% Shoots with necrosis	25	70	70	60	75		P < 0.05

**Data were recorded on the 30 th day and presents an average of 10 replicates per treatment. Different lowercase letters in a row above following any two mean values indicate that these two means are significantly different at P = 0.05 level of significance according to the Student's t-test. Values in brackets show the standard errors of the mean values.*

At the end of the incubation period (30 days) the organic potential of the cultured explants was affected significantly by all types of cytokinins tested (Table 15). Visible shoot elongation and proliferation was observed within 5 days of setting up cultures. One week after inoculation in

some cultures some shoots started to exhibit tip necrosis. The frequencies of necrotic shoots varied significantly with the different cytokinins ($P < 0.05$). Four weeks after inoculation 70%, 70%, 60%, 75% and 25% of cultured explants exhibited shoot tip necrosis on the medium supplemented with BAP, K, 2iP and Zea and growth regulator-free medium in turn. Explants on the control medium did not produce new shoots, or maintained only one shoot per explant and the frequency of the necrotic shoots was lowest (25%). Apart from the cytokinin Zea, all other cytokinins induced callus production at the base of shoots. Callus produced was fairly uniform, which was hard, green to creamy white colour with some red pigmentation. Significant differences were observed in mean shoot length, mean shoot number, mean leaf no and mean bud no between the tested treatments (Table 15). In particular, in terms of shoot length, there was a highly significant difference between BAP and control treatment ($P < 0.001$). However, there were no differences among cytokinins. In general, there were differences between all tested cytokinins in respect of shoot no, leaf no and bud no. BAP and K were superior to 2iP and Zea, whereas 2iP was superior to Zea in this respect. The most effective cytokinin for bud induction, from all cytokinins tested was BAP, while K was as effective as the BAP. There was just a marginal difference between BAP and K. In the case of shoot number, in the growth of cultures on individual cytokinin media, BAP and Kin were superior to 2iP and Zea whereas 2iP was superior to Zea. As can be seen from the recorded information above, the most effective cytokinins were BAP and Kin; Zea and 2iP were clearly less effective relative to maintenance of healthy growth and development of the cultures. Consequently, the cytokinin BAP was selected as the basic growth regulator for further use.

3.2.2.2 : Effects of BAP alone on shoot growth and number

In this experiment the effects of seven concentrations (1, 2, 3, 4, 5, 6 and 8 mgl^{-1}) of BAP on multiplication of four-times-subcultured seedling materials excised from the axenic stock clone of *P. vera* were examined. All cultures were incubated under conditions as described in section 3.2. After 30 days, the mean number of the shoots, the mean length of the shoots, the percentage of explants showing apical necrosis and the percentage of explants produced callus were measured. All data related to apical necrosis and callus correspond to visual observations.

In all BAP treatments shoot formation was observed within 10 days of explanting of the apical tips. On MS medium, highly significant differences were observed in both mean shoot number and mean shoot length on day 30 between the tested concentrations of BAP (Table 16). Explants on medium supplemented with 2, 3 and 4 mgl^{-1} BAP produced the most shoots. The maximum shoot number was achieved at 3 and 4 mgl^{-1}, but was not significantly different from the number obtained at 2 mgl^{-1} BAP. Therefore BAP concentrations between 2 and 4 mgl^{-1} appear to be optimal for promoting shoot multiplication. The results (Table 16) also confirmed

that 2, 3 and 4 mgl^{-1} BAP were almost equally effective in promoting shoot length of cultures. No significant differences in means were obtained with 2, 3 and 4 mgl^{-1} BAP. Significantly shorter and fewer shoots were produced when less than 2 mgl^{-1} BAP or more than 4 mgl^{-1} BAP was used.

As with shoot proliferation, BAP affected the levels of necrosis. Poor growth or abnormal shoots was very common at high concentrations of BAP. The appearance of apical necrosis in all tested concentrations of BAP started within 10 days after incubation. There was not a positive correlation between the tested BAP concentrations and shoots showing apical tip necrosis. However, lower levels of apical necrosis were observed in cytokinin-free medium, showing clearly that promotion of apical necrosis or vitrification is the result of excess BAP in the medium. Four weeks after incubation, 32%, 33%, 28%, 23%, 30%, 27% and 23% of proliferated shoots exhibited shoot tip necrosis on media supplemented respectively with 1, 2, 3, 4, 5, 6, and 8 mgl^{-1} BAP. The frequencies of necrotic shoots did not vary significantly with the different concentrations of BAP.

Table 16 : The effects of BAP on shoot growth and number of cultured explants from seedlings of *P. vera* on media containing different concentrations of BAP.

Treatments (BAP mgl^{-1})	1	2	3	4	5	6	8	F test (79 df)	χ^2 test (7 df)
Mean shoot length (mm)	1.8 b (0.2)	3.3 c (0.3)	3.5 c (0.4)	3.3 c (0.5)	2.5 d (0.2)	2.0 b (0.2)	1.9 b (0.2)	P < 0.001	
Mean shoot no	7.8 ac (0.9)	12.0 b (1.1)	12.7 b (0.8)	12.7 b (1.0)	8.6 a (0.9)	5.3 c (1.0)	3.0 cd (0.6)	P < 0.001	
% Shoots with necrosis	32	33	28	23	30	27	25		N.S.**
% Explants with callus	20	40	30	50	30	30	20		P < 0.01

**Data recorded on the 30 th day and presents an average of 10 replicates per treatment. ** indicates that there is no significant difference. Different lowercase letters in a row above following any two mean values indicate that these two means are not significantly different at P = 0.05 level of significance according to the Student's t-test. Values in brackets show the standard errors of the mean values.*

The apical necrosis was lowest in medium supplemented with 4 mgl^{-1} BAP. There were significant differences in callus production between applied treatments (Table 16, P < 0.01). All tested treatments induced callus production at the base of shoots. The callus produced was fairly uniform, hard and green to creamy white in colour, with some red pigmentation. 30 days after incubation 20%, 40%, 30%, 50%, 30%, 30% and 20% of cultured axillary shoots produced callus on the medium supplemented with 1, 2, 3, 4, 5, 6, and 8 mgl^{-1} BAP respectively. From the experiment, it appears that, as with shoot proliferation, BAP erratically affected the levels of apical necrosis. The general appearance and growth of shoots were best on medium with 3 and 4 mgl^{-1} BAP. In consequence, 3 mgl^{-1} BAP was selected as the minimal level of BAP for shoot multiplication. In the next experiment, the effect of combining BAP with another cytokinin, and subsequently with an auxin, on shoot growth and proliferation of seedlings will be investigated,

with the objective of inducing maximal shoot multiplication in combination with healthy-looking shoots.

3.2.2.3 : Combined effect of cytokinins

In the previous experiment the optimum concentration of BAP for shoot multiplication was determined for explants of seedling pistachio *in vitro*. The following experiment investigates the effect of combinations of cytokinins on shoot growth and proliferation of six-times-subcultured axillary buds. Mixtures of PGRs were applied by incorporating in agarified medium the following combinations of PGRs (mgl^{-1}):

a- BAP at 3 mgl^{-1}

b- BAP at 3 mgl^{-1}: 2iP at 0.5 mgl^{-1}

c- BAP at 3 mgl^{-1}: TZD at 0.5 mgl^{-1}

d- BAP at 3 mgl^{-1}: 2iP at 1 mgl^{-1}

e- BAP at 3 mgl^{-1}: TZD at 1 mgl^{-1}

f- BAP at 3 mgl^{-1}: 2iP and TZD at 0,5 mgl^{-1}

g- BAP:2iP:TZD at 1 mgl^{-1}.

In this experiment the combined effects of cytokinins (the above treatments) on growth and multiplication of four-times-subcultured seedling materials excised from the axenic stock clone of *P. vera* were examined. All cultures were incubated under conditions as described in section 3.2. After 30 days, the mean number of the shoots, the mean length of the shoots, the total bud number, the total shoot number, the maximum shoot length and the percentage of explants showing apical necrosis were measured (Table 17).

The results obtained (Table 17) revealed significant differences in shoot number, shoot length and bud number between those that had all undegone treatments. The combination of BAP (3 mgl^{-1}) with 2iP (at 1 mgl^{-1}) (treatment d) induced more shoots and buds than any of the other tested cytokinin mixtures. Overall, treatment g produced poorest results. In respect to the mean shoot length the treatments d and f were superior to the rest of the treatments tested. Treatments a, b, c and d were clearly the more effective mixtures for proliferation with more shoots than other treatments . In terms of bud no, the treatments a, b, and d proved superior to treatments e, f, and g. There was a significant difference between treatment d and the rest of the treatments at 0.05 significance level ($P < 0.05$) in respect to the bud number. The numbers of necrotic shoots with strap-like leaves decreased when explants were cultured on the mixture of cytokinins (Table 17). Under standard conditions for 30 days. The frequency of necrotic shoots varied significantly with the different mixtures of plant growth regulators (Table 17). Only the 28% of the proliferating shoots were vitrified on media containing 3 mgl^{-1} BAP. However, at the combination of BAP (3 mgl^{-1}) and of the cytokinins tested the percentage of the necrotic shoots

was higher than the only cytokinin BAP applied treatment (the treatment a). As in previous experiments, in some treatments, light green callus growth was observed on the basal end of nodal segments within 30 days. From the experiment it would appear that the use of BAP (3 mgl^{-1}) with 2iP (1 mgl^{-1}) (treatment d) was the best of those tested, for optimal pistachio shoot growth and multiplication. Subsequent experiments will investigate the optimum concentration and combination of BAP and one of the auxins required for maximum multiple axillary shoot development from excised nodal segments while avoiding excessive callus and abnormal growth will also be investigated further in subsequent experiments.

Table 17 : Effects of mixtures of cytokinins on growth and proliferation of 5 times subcultured *P. vera* seedling material*.

Treatments	a	b	c	d	e	f	g	F test (83)	χ^2 test (6 df)
Mean shoot number	3.8 a (0.3)	3.4 a (0.6)	2.6 a (0.7)	4.0 a (0.4)	1.3 b (0.2)	1.0 b (0.1)	1.0 b (0.1)	($P < 0.05$)	
Total shoot number	46	41	32	49	16	13	13		
Mean shoot length (mm)	26.8 b (1.6)	29.7 b (1.6)	27.9 b (1.5)	33.1 a (2.4)	26.6 b (0.8)	33.4 a (0.4)	19.4 c (0.8)	($P < 0.05$)	
Mean bud number	10.8 b	10.7 b	8.2 b	16.8 a	5.8 c	5.2 c	5.4 c	($P < 0.05$)	
Total bud number	130	129	98	202	70	63	65		
Max. shoot length (mm)	38	52	42	59	50	41	29		
Shoots with necrosis (%)	28	43	41	43	12	30	46		($P < 0.05$)

**Data recorded on the 30th day and presents an average of 12 replicates per treatment. Different lowercase letters in a row above following any two mean values indicate that these two means are not significantly different at P = 0.05 level of significance according to the Student's t-test. Values in brackets show the standard errors of the mean values.*

3.2.2.4 : Inclusion of auxins with applied cytokinins

Using 3 mgl^{-1} BAP as the control, the effects of three concentrations (0.05, 0.1 and 0.2 mgl^{-1}) of each of three auxins (NAA, 2,4,5-T and IBA) in addition to 3 mgl^{-1} BAP on seven times subcultured seedling materials were examined. Mixtures of PGRs were applied by incorporation in agar-solidified MS medium the following combinations of PGRs:

1- BAP at 3 mgl^{-1}
2- BAP at 3 mgl^{-1}: NAA at 0.05 mgl^{-1}
3- BAP at 3 mgl^{-1}: NAA at 0.1 mgl^{-1}
4- BAP at 3 mgl^{-1}: NAA at 0.2 mgl^{-1}
5- BAP at 3 mgl^{-1}: 2,4,5-T at 0.05 mgl^{-1}
6- BAP at 3 mgl^{-1}: 2,4,5-T at 0.1 mgl^{-1}
7- BAP at 3 mgl^{-1}: 2,4,5-T at 0.2 mgl^{-1}
8- BAP at 3 mgl^{-1} : IBA at 0.05 mgl^{-1}
9- BAP at 3 mgl^{-1}: IBA at 0.1 mgl^{-1}

10- BAP at 3 mgl^{-1}: IBA at 0.2 $mg\ l^{-1}$.

Media were solidified with agar (0.7%) and all cultures were incubated under conditions as described in section 3.2. The mean number of the shoots, the mean length of the shoots, the mean number of bud, the percentage of explants showing apical necrosis and the percentage of explants produced callus were measured (Table 18).

In all treatments axilliary shoot formation was observed after 10 days incubation of nodal segments and production was measured at 30 days (Table 18) for all treatments. Highly significant differences were observed in mean shoot length, mean shoot number and mean bud number between the tested treatments (Table 18, $P < 0.001$). Treatments 1, 3, 4, 8, 9 and 10 produced significantly more shoots than treatments 2, 5, 6 and 7. This suggests that there is not a sharp sensitivity in *P. vera* to the addition of auxins.

Table 18 : Effect of exogenous auxins (NAA, 2,4,5-T and IBA) adding a cytokinin (BAP) on the morphogenetic responses of cultured explants of *P. vera* seedlings after 4 weeks on MS medium*.

	BAP	BAP + NAA			BAP +2,4,5-T			BAP + IBA			F test (115 df)	χ^2 test (9 df)
Treatments	1	2	3	4	5	6	7	8	9	10		
Mean shoot length (mm)	27.9 a (1.0)	25.3 a (2.7)	25.7 a (2.2)	24.5 ab (1.4)	22 bc (1.2)	20.7 c (0.6)	16.0 d (0.5)	26.1 a (1.1)	30 ae (1.8)	25.8 ab (1.2)	(P < 0.001)	
Mean shoot no	3.0 a (0.3)	1.9 b (0.2)	3.6 a (0.3)	2.6 a (0.4)	2.0 b (0.3)	1.7 b (0.2)	1.5 c (0.2)	2.7 a (0.2)	2.9 a (0.3)	2.6 a (0.3)	(P < 0.001)	
Mean bud no	11.5 a (0.7)	8.4 b (1.4)	7.3 b (0.5)	10.5 b (2.6)	4.1 c (0.4)	6.3 b (1.0)	3.6 d (0.3)	10 b (1.2)	12.8 ab (0.8)	9.5 b (1.1)	(P < 0.001)	
Shoots with necrosis (%)	37	47	48	56	58	42	55	48	31	40		N.S**
Explants with callus (%)	10	20	50	30	20	30	25	35	25	30		(P < 0.05)

**Data recorded on the 30 th day and presents an average of 20 replicates per treatment.* ** indicates that there is no significant difference. Different lower case letters in a row above followed by any two mean values indicate that these two means are significantly different at P = 0.05 level of significance according to the Student t-test. Values in brackets show the standard errors of the mean values.*

The length of shoots produced by BAP alone , was significantly greater than in some treatments, i.e. 5, 6, and 7 but not in others, e.g. 2-4 and 8-10. With the exception of IBA the inclusion of auxins inhibited bud production, 2,4,5-T being more inhibitory than NAA. The best responses in respect to shoot length, and mean bud number were noted in a combination of BAP (3 mgl^{-1}) and IBA (0.1 mgl^{-1}) (Table 18) with a mean shoot length of 30 (mm) ± 1.8 and with a mean bud number of 12.8 ± 0.8 per explant on the 30th day of culture.

All auxin treatments produced greater callus than the control. In all treatments except the BAP alone, green callus growth with some red pigmentation was observed on the basal end of nodal segments within 30 days. Callus production from shoot explants was variable depending on the level of plant growth regulators. The inclusion of exogenous auxins (NAA, 2,4,5-T and IBA),

even at the low level applied in this experiment, with the cytokinin BAP favoured increased in callus proliferation rather than the production of adventitious buds on cultured explants. The frequencies of explants producing calluses varied significantly with the different treatments ($P < 0.05$). However, there was no obvious pattern in the frequencies of explant produced callus. 30 days after incubation 10%, 20%, 50%, 30%, 20%, 30%, 25%, 35%, 25% and 30% of explants produced callus on the treatments 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, respectively. Apical necrosis was evident in all treatments but none induced significantly more or less than any another (Table 18). An optimum necrosis level of 58% being obtained media with 3 mgl^{-1} BAP plus 0.01 mgl^{-1} 2,4,5-T after 30 days of culture.

A comparison of BAP alone and combinations of BAP and auxins indicated that there were significant differences between the combined treatments, but none of the combined auxin/BAP treatments were superior to BAP alone. From the experiment, it is concluded that large callus growth and a high level apical necrosis appeared to envelop the axilliary bud preventing proliferation of shoots within the 30-day period. The addition of auxins did not alter the effectiveness of BAP and even the majority of explants had a different pattern of response to combined treatments. The inclusion of exogenous auxins, even at the low level applied in this study resulted in a significant increase in callus proliferation rather than the production of adventitious shoots on cultured nodal bud segments (Table 18). Therefore, the inclusion of auxins will not be used to modify a basal medium in further studies.

3.2.3 : Effects of basal media on shoot growth and multiplication

In this experiment, the effects of different culture media on shoot number, shoot length, callus production and necrotic shoot production were investigated. Shoot tip explants consisting of a nodal and apical tip were cultured from 8 times subcultured-cloned stock material. Explants were plated on agar-solidified media containing 3% sucrose, 0.7% agar, and pH was adjusted to 5.7 with 0.1 N NaOH. The following media at the suggested levels, supplemented with 3 mgl^{-1} BAP and 1 2iP were tested:

a- Murashige and Skoog Basal Medium with Gamborg's vitamins: MS (Sigma, M 0404)
b- McCown's Woody plant Basal Salt Mixture: WP (Sigma, M 6774)
c- Schenk and Hildebrandt medium: SH (Imperial, 9-650-35)
d- Gamborg's B-5 : G-5 (Sigma, G 5768)
e- Anderson's Rhodendron Basal salt Mixture Rhodendron: A (Sigma, A 2670).
All cultures were incubated under conditions as described in section 3.2.

Significant differences were observed in both the mean shoot number and the mean shoot length between the five media types tested at suggested concentrations (Table 19). After 30

days of explanting, the best response in terms of number and length of healthy shoots was obtained with MS, and the poorest response with the A medium (Table 19). Apart from the MS, in the rest of the media, the length of the induced shoots were not significantly different. Significantly fewer shoots were produced on SH medium than on the WP, G-5 and A media. There were highly significant differences in the frequencies of explants producing callus between the five media sources tested (Table 19, $P < 0.01$). The shoots grown on MS also produced the lowest percentage of the calluses at the basal end of the explants; this occurred in 15% of the cultures as compared with 50%, 65%, 30% and 20 % cultures for WP, SH, G-5 and R media, respectively. Significantly different rates of necrotic shoots were obtained with all four media sources tested at the suggested levels (Table 19). Few necrotic shoots were produced by cultures on MS medium. Necrosis levels were reduced from 30% to 8.8% and 9.1% by growing seedlings on full strength G-5 and MS medium, respectively, instead of SH medium after 30 days of culture. Only 9.1% of the proliferated shoots produced necrosis on the MS medium. In general, the appearance was good and growth habits of shoots were better on the MS medium than on other media tested. Therefore, MS medium was chosen for further studies.

Table 19 : Effects of basal culture media on *P. vera* shoot growth and multiplication*.

	WPM	MS	SH	A	G-5	F test (99)	χ^2 test (4 df)
Mean shoot number	2.2 a (0.3)	4.3 b (0.5)	1.5 c (0.1)	1.2 cd (0.1)	2.0 a (0.2)	P < 0.001	
Mean shoot length (mm)	19.5 a (1.0)	26.3 b (1.2)	21.5 a (1.1)	14.3 c (0.6)	19.5 a (1.5)	P < 0.001	
Explants with callus (%)	50	15	65	30	20		P < 0.01
Shoots with necrosis (%)	17.7	9.1	30	24	8.8		P < 0.05

**Data recorded on the 30 th day and presents an average of 20 replicates per treatment. Different lower case letters in a row above followed by any two mean values indicate that these two means are significantly different at P = 0.05 level of significance according to the Student t-test. Values in brackets show the standard errors of the mean values.*

Comparisons of the relative concentrations of components in the above media are summarised in Table 20. From the experiment, the data presented in Table 19 indicate that the response of subcultured nodal bud segments was clearly better with the MS medium than with other media, supporting the conclusion of the studies in the subsequent section with the MS medium seemed to be most suitable for organogenesis. However, the differences in performance of shoot cultures which were observed in the current experiment raised the question as to which factors in MS medium support good shoot growth and proliferation. In view of this, in a subsequent experiment an investigation was carried out to confirm the superiority of MS medium over WP medium by adding missing nitrogen compounds (ammonium nitrate and potassium nitrate) in WP medium. Cultures were incubated under standard conditions for 30 days. After 30 days,

mean shoot number and length, the percentage of explants showing necrosis and the percentage of explants inducing necrosis was recorded.

Table 20 : Mineral components (mgl^{-1}) of the culture media used for shoot multiplication of *P. vera.*

Contents	MS	WPM	SH	G-5	A
Ammonium sulphate	-	-	-	134.0	-
Ammonium nitrate	1650.0	400.0	-	-	400.0
Boric acid	6.20	6.20	5.0	3.0	6.20
Calcium chloride anhydrous	332.20	72.50	200	113.24	332.2
Calcium nitrate (vacuum dried)	-	386.0	-	-	-
Cobalt chloride hexahydrate	0.0250	-	0.1	0.0250	0.025
Cupric sulphate pentahydrate	0.0250	0.250	0.2	0.0250	0.025
Disodium EDTA dehydrate	37.260	37.30	20	37.250	74.50
Ferrous sulphate heptahydrate	27.80	27.80	15	27.850	55.70
Magnesium sulphate anhydrous	180.70	180.7	400	122.09	180.7
Manganese sulphate	-	22.30	-	-	-
Manganese sulphate monohydrate	16.90	-	10.0	10.0	16.90
Myo-Inositol	100.0	-	1000	-	-
Nicotinic acid (Free acid)	1.0	-	50	-	-
Potassium iodide	0.830	-	1.0	0.750	0.30
Potassium nitrate	1900.0	-	2500	2500.0	480.0
Potassium phosphate monobasic	170.0	170.0	-	-	-
Potassium sulphate	-	990.0	-	-	-
Pyridoxine hydrochloride	1.0	-	0.5	-	-
Sodium molybdate dehydrate	0.250	0.250	300	0.250	0.250
Sodium phosphate monobasic	-	-	-	130.0	330.6
Thiamine hydrochloride	10.0	-	5.0	-	-
Zinc sulphate heptahydrate	8.60	8.60	1.0	2.0	8.60

Table 21 : Effects of culture media on *P. vera* shoot growth and multiplication*.

	MS	WP	WP + Missing nit. compounds	F test (59 df)	χ^2 test (2 df)
Mean shoot number	3.8 a (0.3)	2.7 b (0.2)	3.0 ab (0.2)	P < 0.05	
Mean shoot length (mm)	23.2 a (0.9)	20.6 b (0.5)	21.4 ab (0.6)	P < 0.05	
Explants with callus (%)	40	30	25		N.S.**
Shoots with necrosis (%)	22.8	14.5	23.3		N.S.**

**Data recorded on the 30 th day and presents an average of 20 replicates per treatment. ** indicates that there is no significant difference. Different lower case letters in a row above followed by any two mean values indicate that these two means are significantly different at P = 0.05 level of significance according to the Student's t-test. Values in brackets show the standard errors of the mean values.*

The results (Table 21) showed again that MS medium had a real significant effect on shoot growth and proliferation. Explants on MS produced more and longer shoots than those on WP media and WP supplemented with nitrogen compounds (1250 mgl^{-1} ammonium nitrate and 1900 mgl^{-1} potassium nitrate) present in MS but not present in WP medium. The difference between MS and WP was significant but between MS and nitrogen-supplemented WP medium was not

significant in terms of mean shoot number. There were no significant differences in the frequencies of explants producing calluses and necrotic plants between the tested treatments (Table 21). After 30 days incubation under standard conditions, only 25% of explants cultured on WP medium with the added nitrogen compounds showed necrotic shoots as compared with 40% and 30% on MS and WP media, respectively. The most noteworthy result obtained in this experiment was the confirmation of the value of the MS medium.

3.2.3.1 : Effects of mineral medium strength on shoot growth and proliferation

Nodal bud segments (subcultured nine times) were cultured on MS medium supplemented with 3 mgl^{-1} BAP and 1 mgl^{-1} 2iP. In order to examine the effect of medium strength on shoot growth and multiplication, the concentration of nutrients in MS medium was varied from half to full and double strength. All cultures were incubated under conditions as described in section 3.2. After 30 days, the mean number of the shoots, the mean length of the shoots, the mean number of bud, the percentage of explants showing apical necrosis and the percentage of explants produced callus were measured (Table 22).

The effects of half, full and double strength nutrients in the culture medium upon viability are shown after 30 days of incubation (Table 22). The medium strength used significantly affected the mean shoot number and mean shoot length that were produced. Explants from plantlets cultured on half strength medium regenerated significantly less shoot length than those from plantlets cultured in full and double strength media, whereas the use of double strength nutrient did not have any beneficial effect on shoot length. The strength of the medium used also significantly affected the mean shoot number of the resulting axillary shoot cultures. Explants incubated on the full strength medium had more shoots and more buds per explant than those on the half and double strength media. Reducing the concentration of nutrient did not have any beneficial effect on shoot growth, whereas raising the concentration of nutrients had a negative effect on shoot number and bud number, which were significantly lower on double-strength than on the full strength medium. The frequencies of explants producing callus did not vary significantly with the different strength of the medium (Table 22). Explants produced callus in 40% and 45% of cultures in either half or full strength MS medium but explants on full MS medium shoots doubled their initial number during 30 days of culture. The observed frequency of the number of proliferated shoots in which necrotic shoots occurred was compared using a chi-square test. There was not a significant difference between those that had undergone treatments ($P > 0.05$, Table 22).

The frequencies of explants producing callus did not vary significantly with the different strength of the medium (Table 22). Explants produced callus in 40% and 45% of cultures in either half or

full strength MS medium but explants on full MS medium shoots doubled their initial number during 30 days of culture. The observed frequency of the number of proliferated shoots in which necrotic shoots occurred was compared using a chi-square test. There was not a significant difference between those that had undergone treatments (P > 0.05, Table 22).

Table 22 : Effect of strength medium on the growth and multiplication of cultured explants on *P. vera* seedlings after 30 days of incubation*.

Treatments	Half strength	Full strength	Double strength	F test (59 df)	χ^2 test (2 df)
Mean shoot length (mm)	19.6 a (0.7)	22.9 b (1.2)	23.8 bc (1.2)	P < 0.05	
Mean shoot no	1.9 a (0.1)	2.2 ab (0.1)	1.6 ac (0.1)	P < 0.05	
Mean bud no	6.8 a (0.8)	7.4 ab (0.7)	4.5 bc (0.5)	P < 0.05	
Shoots with chlorisis (%)	17.9	11.1	19.5		N.S.**
Explants with callus (%)	40	45	75		N.S.**

**Data recorded on the 30 th day and presents an average of 20 replicates per treatment. ** indicates that there is no significant difference. Different lower case letters in a row above followed by any two mean values indicate that these two means are significantly different at P = 0.05 level of significance according to the Student t-test. Values in brackets show the standard errors of the mean values.*

The frequencies of explants producing callus did not vary significantly with the different strength of the medium (Table 22). Explants produced callus in 40% and 45% of cultures in either half or full strength MS medium but explants on full MS medium shoots doubled their initial number during 30 days of culture. The observed frequency of the number of proliferated shoots in which necrotic shoots occurred was compared using a chi-square test. There was not a significant difference between those that had undergone treatments (P > 0.05, Table 22).

3.2.4 : Effects of photoperiod on growth and development of pistachio shoot cultures

Nodal bud segments were grown on MS medium supplemented with 3 mgl^{-1} BAP plus 1 mgl^{-1} 2iP. Cultures were incubated for 30 days under the following environmental conditions: 25°C/continuous photoperiod, 25°C/complete darkness and 25°C/18-h photoperiod.

Examples of influence response present for both the average shoot number and the average number of shoot length are shown in Table 23. The results obtained in the current study indicated that incubation of pistachio shoot cultures at 25°C/continuous photoperiod improved shoot growth and proliferation when compared with 25°C/complete darkness and 25°C/18 h, though not significantly except in the case of the mean number of the shoot length produced (Table 23, P < 0.01).

After 30 days on the shoot formation medium, the maximum shoot length per explant was obtained under the continuous light treatment. The average length of shoots was 35.1 ± 2.2 and the average number of shoots was 2.1 ± 0.1 under continuous light (mean ± SE). In darkness, the two parameters were 22.6 ± 1.6 and 2.0 ± 0.1, respectively. In 18-h photoperiod, the parameters were 1.9 ± 0.1 and 21.4 ± 0.1, respectively.

Table 23 : Effects of photoperiod on growth and development of *P. vera* shoot cultures*.

	Continuously light	Completely dark	18 hs photoperiod	F test (59 df)	χ^2 test (2 df)
Mean shoot number	2.1 a (0.1)	2.0 a (0.1)	1.9 a (0.1)	P > 0.05	
Mean shoot length (mm)	35.1 a (2.2)	22.6 b (1.6)	21.4 b (1.1)	P < 0.001	
Explants with callus (%)	25	35	15		N.S**.
Shoots with necrosis (%)	11.9	17.0	5.2		N.S**.

**Data recorded on the 30 th day and presents an average of 20 replicates per treatment.. ** indicates that there is no significant difference. Different lower case letters in a row above followed by any two mean values indicate that these two means are significantly different at P = 0.05 level of significance according to the Student's t-test. Values in brackets show the standard errors of the mean values.*

Concerning shoot number, no significant differences in means were obtained between the treatments although the continuous light treatment gave the best overall mean results. Significant differences were not observed in the frequencies of shoots becoming necrotic and explants producing callus due to the different photoperiod (Table 23). The highest shoot number was obtained from the continuous light treatment but this was not significantly different from those observed in the total darkness (24-h) and 18-h light treated treatments. In the present study, the results obtained suggest that incubation of cultures under continuous light is suitable for growth and health proliferation of pistachio shoots *in vitro*. In the following experiment, the effect on health shoot formation of irradiation with different light density will be further investigated.

3.2.5 : The influence of light intensity on shoot growth and development of *P. vera* seedling material

Nodal bud segments subcultured 10 times were cultured on the full strength MS medium for 30 days at 25°C with a continuous photoperiod. Cultures were maintained under three light intensities (20 ± 5, 40 ± 5 and 80 ± 5 µmol m^{-2} s^{-1}), in the presence of cytokinins (3 mgl^{-1} BAP+ 1 mgl^{-1} 2iP), were evaluated for shoot length and shoot production. The photosynthetic radiation for the light intensity evaluation was supplied by cool-white fluorescent tubes (or lamps). Desired

light intensities were obtained by varying the distances between cultures and the light source, and were maintained constant during the continuous photoperiod. All cultures were incubated under conditions as described in section 3.2.

Table 24 : The influence of light intensity on shoot growth and development in *P. vera* cultures*.

	20 ± 5 μmol m^{-2} s^{-1}	40 ± 5 μmol m^{-2} s^{-1}	80 ± 5 μmol m^{-2} s^{-1}	F test (55 df)	χ^2 test (2 df)
Mean shoot number	1.5 a (0.1)	2.0 b (0.1)	1.4 a (0.1)	P < 0.05	
Mean shoot length (mm)	20.6 a (0.8)	36.0 b (3.0)	20.1 a (1.1)	P < 0.001	
Explants with callus (%)	25	35	20		N.S.**
Shoots with necrosis (%)	23	20	56		P < 0.01

**Data recorded on the 30 th day and presents an average of 20 replicates per treatment. ** indicates that there is no significant difference. Different lower case letters in a row above followed by any two mean values indicate that these two means are significantly different at P = 0.05 level of significance according to the Student's t-test. Values in brackets show the standard errors of the mean values.*

Statistical evaluation of the data revealed that there was a significant difference in the multiplication rate of the explants under different light intensity (Table 24). The highest light intensity (80 μmol m^{-2} s^{-1}) resulted in lowest shoot number and shoot length. Light intensities below 80 μmol m^{-2} s^{-1} supported shoot growth and development. There was no difference in shoot number and shoot length at 20 and 80 μmol m^{-2} s^{-1} but at 40 μmol m^{-2} s^{-1} shoot number and shoot length was significantly greater than at 20 and 80 μmol m^{-2} s^{-1}.

The chi-square test of the data indicated that significant differences were found between the shoots showing apical tip necrosis under different light intensities (Table 24). Cultures kept at 40 μmol m^{-2} s^{-1} produced the lowest number of shoots showing apical necrosis. No significant differences were noted in callus production between the different light intensity treatments. It may be concluded that light intensity (as evaluated from 20 to 80 μmol m^{-2} s^{-1}) had significant effects on shoot growth and proliferation on cultures of *P. vera* in this study. It also inhibited new shoot formation and development in 20% of the explants at the high intensity treatment (80 μmol m^{-2} s^{-1}).

3.2.6 : Influence of age of donor seedlings on shoot growth and proliferation

To examine the relationship between the initiation potential of juvenile seedling materials and their degree of differentiation a study was performed on the influence of BAP at 3 mgl^{-1}, and 2iP at 1 mgl^{-1} on the potential of excised apical shoot tips from pistachios of different age classes (6, 9 and 16 month-old seedlings). The shoot materials were surface-sterilised as explained in

section 3.1.9. The explants were cultivated on agar-solidified MS medium containing 3% sucrose and the pH of the medium was adjusted to 5.7 before autoclaving. Cultures were incubated under standard conditions for 30 days.

Initiation of multiple shoots was observed in all the age classes after 30 d in culture (Table 25). Seedlings of different age classes did not display a range of variation in their response. The explants cultured in MS medium responded by enlarging and producing generally 2 new shoots per explant attaining a height of 2-4 cm within 30 days.

Table 25 : Effect of seedlings of different age classes on *in vitro* multiple shoot initiation from apical explants of *P. vera**.

Time (month)	Percentage of explants which responded	Average no of shoots per culture ± SE*	Average shoot length (mm) ± SE*
6	84.4	2.1 ± 0.2 a	21.3 ± 1.1 b
9	91.7	2.3 ± 0.2 a	20.2 ± 0.9 b
16	75.0	1.6 ± 0.2 a	18.2 ± 0.7 b
χ^2 test (1 df)	N.S.**		
F test (38)		N.S.**	N.S.**

*Data recorded on the 30 th day and represents an average of 12 replicates per treatment. ** Different lower case letters in a column above followed by any two mean values indicate that these two means are significantly different at P = 0.05 according to the Student's t-test.*

The maximum percentage of explants responding and the greater number of the shoots were obtained from 9 month-old seedling explants (Table 25). However, no significant differences in mean values were obtained with the different age classes. Lower rates of shoot production were obtained on the 16 month-old explants than on the other tested age classes. From the experiment it should be concluded that the potential for inducing adventitious shoots on cultured explants did not significantly increase with age of the explanted seedlings. It should also be noted that among the seedlings up to 16 months the rate of initiation declined.

3.2.7 : Bud multiplication

For bud multiplication, the axillary shoots including 3 buds (subcultured 9 times) were recultured on MS medium supplemented with 3% sucrose, 3 mgl^{-1} BAP and 1 mgl^{-1} 2iP, where a final account of the number of induced primary shoots and the number of the secondary buds per 20 cultured explants was made. To examine the possibility of increasing the production of axillary buds through surgical removal of the apical tips, so as to overcome the apical dominance over axillary buds and vitrification, the explants were cultured with or without apical tips. Cultures were incubated for 30 days under standard conditions.

Table 26 : Effects of excision of the apical bud for cultured explants of *P. vera* on the production of primary shoots and secondary buds on a modified MS medium.

Type of cultured explants	Mean no of primary shoots ± SE	Mean height of longest primary shoots (mm) ± SE	Mean no of secondary buds ± SE	Primary shoots with necrosis (%)
Explants with apical tips	2.8 ± 0.1 a	6.8 ± 0.4 a	14.2 ± 0.7 a	46
Explants without apical tips	4.2 ± 0.3 b	8.7 ± 0.4 b	19.5 ± 1.1 b	33
χ^2 test (1 df)				N.S.*
F test (39)	P < 0.01	P < 0.01	P < 0.01	

*Data recorded on the 30 th day and represents an average of 12 replicates per treatment. *Different lower case letters in a column above followed by any two mean values indicate that these two means are significantly different at P = 0.05 level of significance according to the Student's t-test.*

Significant differences were observed in mean shoot number, mean height and mean number of secondary buds between the two types of cultured explants (Table 26). The explants cultured with apical tips produced a lower number of primary shoots, secondary buds and shorter shoots. In the two types of explants tested a considerable number of necrotic axillary shoots was observed at the apical tips. When explants were cultured without apical tips, the formation of necrotic shoots at the apical tips was considerably reduced. Nevertheless, there was not a significant difference in necrotic shoot production between the two types of explants cultured.

3.2.8 : Discussion

This study showed that BAP and K were more suitable than 2iP and Zea for multiple shoot proliferation from *P. vera* seedling material, generally continuing the accepted findings of others (e.g. Barghchi and Alderson 1983a and Busmante-Garcia 1984) (Table 15). However, the results presented here reveal that not only BAP alone but also K alone is as effective as BAP in bringing about shoot formation in cultures of pistachios. This study also revealed that BAP is more potent, in terms of induction of bud and shoot number than the other cytokinins tested. Excessive callus growth was observed at the base of shoots in all tested cytokinins. Similar results were obtained by Barghchi and Alderson (1983a). There was a high level of apical necrosis in cultures of all tested cytokinins. Although BAP is the most commonly used plant growth regulator source in culture media and is commonly used for pistachio by other workers (Barghchi 1982, Busmante-Garcia 1984 and Abousalim 1990), my results indicate that improvements in multiplication rate and explant size can be realised by using K or BAP.

After four weeks of incubation, of the BAP levels tested, 3 mgl^{-1} BAP was the most suitable in promoting shoot multiplication of cultures. The greatest number of shoots produced (BAP 4 mgl^{-1}, Table 16) is significantly greater than that of the control ($P < 0.001$) but there were no significant differences in means obtained with either 2, 3 or 4 mgl^{-1} BAP. Different responses to BAP have been obtained with *P. vera* seedling materials by several workers. My results are in agreement with those obtained by those workers (Barghchi and Alderson 1983 and Abousalim 1990) who found that a medium containing 4 mgl^{-1} BAP was optimal for seedling explant obtained from 10-day-old aseptically germinated seeds. Grafted plants of one to four-year-old plants were reported to perform better in 2 mgl^{-1} BAP (Martinelli 1988). Similar to the observation reported here, an intermediate result was found when two-month-old plants were cultured in the presence of 3 mgl^{-1} BAP (Bustamante-Garcia 1984). Concerning *P. atlantic,* multiplication was reported to perform better in 0.7 and 1 mgl^{-1} BAP (Martinelli 1988 and Abuosalim 1990), respectively. The erratic results obtained proved very difficult to analyse as there was no logical reason for almost equal percentages of apical necrosis. Apical necrosis levels recorded by Abousalim (1990) (more than 50%) after four to five weeks incubation in similar conditions is comparable to the present results (Table 15).

The combination of BAP (3 mgl^{-1}) with 2iP (1 mgl^{-1}) produced more shoots, buds and taller shoots than TZD (at 0.5 and 1 mgl^{-1}) or 2iP at 0.5 mgl^{-1}. These results are in agreement with those obtained by Abousalim (1990) who found that 2iP had a weaker effect than did BAP in inducing shoot production from *P. vera* seedlings. However, other alternatives for producing better growth with healthier shoots have been reported. Barghchi and Alderson (1983) noted that supplementation of media with either Kin or GA_3 with or without BAP proved not to be beneficial for shoot growth or proliferation of *P. vera.* The results presented in Table 17 showed strong evidence of a real effect of BAP combined with 1 mgl^{-1} 2iP. These results suggest that 3 mgl^{-1} of BAP and 1 mgl^{-1} 2iP resulted in a significant increase in shoot and bud proliferation and in shoot length. The addition of 2iP did alter the effectiveness of BAP alone and there is a different pattern of response to proliferation and multiplication. My results are in agreement with Abousalim's (1990) observations for pistachio seedling material that the combination of BAP (3 mgl^{-1}) with 2iP (0.5 and 1 mgl^{-1}) was the best for shoot multiplication.

BAP+Auxins (NAA, IBA and 2,4,5,-T) proved not to be the best combination, and BAP alone rather than BAP + any of the tested auxins was most effective in promoting axillary bud development, and subsequent multiplication of buds and shoots on axillary shoot explants (Table 18). This was previously not shown to be the case for shoots *in vitro* from seedlings of *P. vera.*

Results obtained in this study revealed that organogenesis on pistachio seedling explants can be influenced by the mineral salt type and levels in the medium (Table 19). Murashige and Skoog

medium (MS) or a modification of this medium have been commonly used for shoot proliferation of *Pistacia* species (Barghchi 1982; Bustamante-Garcia 1984; Martinelli 1988; Abousalim 1990). Barghchi (1985) tested MS medium against WP medium formulae and found no differences between the performances of shoots of *P. vera* when grown on either of the two media. Similar differences in performance to the different media have been reported by Bustamante-Garcia (1984) who tested the response of pistachio tissue to 6 different basal media (B5, Cheng, modified Erikson, Heller, White. and WP medium) in shoot proliferation and found the best response in terms of the number of nodes producing shoots, the number of shoots initiated per segment, and the length of the shoots, with a modified Eriksson liquid medium using filter paper bridges. Abousalim (1990) tested the effects of a range of different macro- and micro-nutrients formulae on shoot proliferation using the media MS, Anderson, Knop and WP medium, supplemented with MS vitamins, and investigated the superiority of MS medium for shoot proliferation of pistachio despite a high level of callus and faint necrosis. In the current study MS medium proved to be superior for pistachio (Table 19).

Perhaps significantly, MS medium is characterised by a high concentration of nitrogen compounds (Table 21). Elsewhere, this has been confirmed for pistachio (Abousalim 1990). Medium strength (as evaluated from half to full and double) had significant effects on shoot length and shoot production on cultures of *P. vera* in this study. Previous work by Barghchi (1983) suggested that medium strength had no effect on shoot production of cultures of *P. vera*, but the half strength MS medium induced a greater number of shoots on explants of (*P. khinjuk*) than the full straight medium evaluated in their study. This suggests that genotypic differences may exist in shoot formation of *Pistacia* explants in response to medium strength in culture.

Mean shoot length was significantly different on MS medium with the different photoperiod (Table 23, $P < 0.01$). After 4 weeks on the shoot formation medium, the maximum length of shoots produced per explants was obtained under the continuous light treatment. Concerning shoot number, no significant differences in means were obtained between each treatment although the continuous light treatment gave the best overall mean results. Significant differences were not observed in the frequencies of shoots becoming necrotic and explants producing callus due to the different photoperiod. Shoots grown under continuous light produced callus in 25% of the cultures as compared with 35% cultures for 18 h treatment. As with callus production, photoperiod affected the levels of vitrification, necrosis was also evident and more than half of the cultures became vitrified on medium maintained for 4 weeks in darkness and leaves produced in darkness were very thin and small. The results obtained suggested that incubating cultures at continuous photoperiods might provide suitable conditions for a better proliferation and growth of seedling material.

3.3 : ESTABLISHMENT OF 50-YEAR-OLD *P. VERA* DERIVED APICAL TIPS OR NODAL BUD SEGMENTS

The methodologies for asexually propagating *P. vera* and other *Pistacia* species by *in vitro* techniques were developed by several workers (Barghchi 1982, Bustamante-Garcia 1984 and Abousalim 1990) using *P. vera* seedling material as a source of developmentally uniform explants. However, when explants of adult material of *P. vera* and other species of the genus *Pistacia* were used, they did not respond to the cultural conditions. Although high survival (85%) was obtained by Abousalim (1990) with explants derived from 30-year-old trees, maintenance of cultures was difficult because of slow decline in rates of growth and increases in the frequency of vitrification and chlorisis.

The following study (3.3.1) was designed to optimise the culture conditions for *in vitro* initiation and multiplication of cultures derived from mature field grown pistachio trees. The effect of the explant type used for the establishment, the position of explants on mother plant, time of the year explant harvested and the influences of plant growth regulators on shoot initiation and multiplication of cultures from excised adult *P. vera* explants was tested with respect to the measured parameters, chosen so to as assess culture initiation and development, the responding rates of cultures, the percentage of contamination, the percentage of necrosis, the survival rates of cultures, the average shoot length and the average bud number.

3.3.1 : Effect of explant type used for initiation

In the present experiment, the effect of explant source used for initiation of pistachio mature material was investigated. Plant material was harvested in mid-June 1994 and surface sterilised apical or nodal segments were cultured on agar solidified medium containing 2 mgl^{-1} BAP 3 gl^{-1} sucrose using the two following methods:

Method I. After sterilisation with 20% sodium hypochlorite, having removed the waxed ends the explants were rinsed through three changes of sterile distilled water. Apices or buds that were 10-15 mm long were cultured onto shoot proliferation medium.

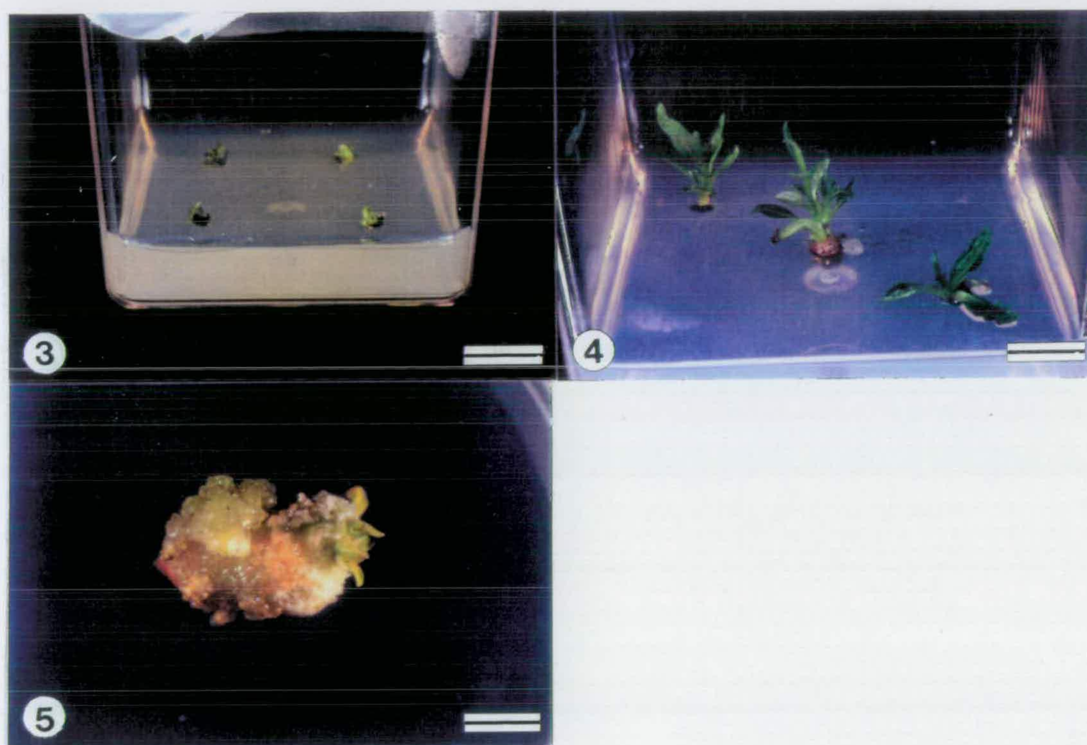
Method II. Meristem tips were cultured onto shoot proliferation medium. After sterilisation with sodium hypochlorite, the explants were rinsed with three changes of sterile distilled water. Having removed the waxed ends and the outer scale leaves, each bud was placed in a sterile Petri dish and the young leaves (bright-green) and scale leaves (red-brown) were carefully removed. Then, the remaining primordia were removed with a very sharp scalpel under a dissecting microscope. The thin meristem was carefully removed from the rest of the explant and transferred onto the surface of the same agar-solidified medium as in the first method.

Table 27 : Effects of methods of culture initiation on establishment of 50-year-old *P. vera* explants*.

	Cont. (%)	Necrotic explants (%)	Medium browning (%)	Survival (%)	Mean shoot length (mm) ± SE
Method I**	40	60	100	60	0
Method II**	10	30	60	90	7.2 ± 0.6
χ^2 test (1 df)	P < 0.05	N.S.	P < 0.01	P < 0.01	
F-test (23 df)					P < 0.001

**Figures presented are the means of 20 replicates observed 35 d of cultures. **Indicates methods described in text. N.S.: Indicates that there is no significant difference at P = 0.05 level of significance according to the Student's t-test.*

The results presented in Table 27 shown for 5 weeks of culture, show that the use of Method II lowered the level of medium browning and resulted in a significant reduction in rates of contamination as compared with Method I.



***Figs. 3-5** : Establishment of meristem tip culture of P. vera. **Fig. 3** : Cultured meristem tip 10 days after culture on MS medium containing 0.5 mgl⁻¹ BAP, bar = 14 mm. **Fig. 4** : Induced pistachio shoots on MS medium after 5 weeks of culture, bar = 7.2 mm. **Fig. 5** : Callus induction on the base of proliferating shoots, bar = 4.5 mm.*

Moreover, the percentage of surviving explants was higher using Method II. The establishment of explants was significantly affected by the method used for culture initiation with 60% and 90%

survival being obtained using apex and meristem tips, respectively. The surviving explants were easily maintained thereafter on the shoot proliferation medium. Although only 10% of the explants were contaminated in Method II, a serious problem of contamination and severe browning was experienced when Method I was used. Browning was observed within 24 h of incubation in Method I, while browning of explants first appeared within 3 days of culture in Method II. Although a high percentage of necrotic explants was observed in Method II there was not a significant difference in the frequencies of necrotic explants between the two methods (Table 27).

Within 10 days of inoculation of the shoot meristem onto the shoot outgrowth medium (Fig. 3), the leaf primordia changed from dark to bright green. In all of the surviving explants, the shoot apices expanded at the base and extended in length to 3-7 mm within three weeks of culture. Outgrowth of the shoot and formation of rolled leaves was observed after 3 weeks of incubation. Younger leaf primordia grew out through the rolled outer leaf. Most of the apical tips did not show proliferation of shoots from excised meristems.

The surviving explants were easily maintained thereafter on the shoot proliferation medium up to 6 months. Multiple shoot development was never observed when the surviving explants were regularly recultured or subcultured on the same or on different media. None of the treatments produced callusing tissues during the first 5 weeks of culture. The maximum percentage of explants producing shoots and the greatest length (7.2 ± 0.5) of the shoot was obtained using 2 mgl^{-1} BAP. The newly formed shoots (Fig. 4), were cut into segments containing one bud and transferred to fresh medium supplemented with 0.5 mgl^{-1} BAP, but in all the surviving proliferating shoots considerable callusing was observed at the base of the explants (Fig. 5) after the third reculture or subculture. Although a high level of survival of regenerating meristem tips was achieved in these cultures, no effective establishment of rapidly proliferating meristem tip cultures could be obtained.

3.3.2 : Effects of position of explants on mother plants

The origins of explants, and in particular their positions on mother plants, have been reported to influence their establishment and growth *in vitro* (Barghchi and Alderson 1985; Abousalim 1990). An experiment was carried out next to investigate whether the degree of juvenility or maturity of an apical meristem depends on its distance (along trunk and branches) from the root-shoot junction. A diagram of the sampled apical meristems' gradients is shown in Figure 6.

Shoots were harvested in early February 1995 and cultured as explained in section 3.3.1 on an MS medium supplemented with 2 mgl^{-1} BAP and cultures were incubated for 5 weeks in

continuous light before being recultured onto a new medium. The reculturing or subculturing was carried out as described in the previous experiment. Explants were harvested from the same branch: a) from far end of the root-shoot junction (apical tips), b) closer part of the root-shoot junction (terminal tips), and c) between them (Figure 6). Levels of survival, the average shoot length and the average bud number was assessed after 5 weeks of culture.

The position of the explants did not significantly influence the survival rate obtained by explants positions a, b and c with 50%, 60% and 75%, respectively. The greatest length of the shoots was obtained using the explants harvested from the position c although differences between the a, b and c harvested explants generally were not significant (Table 28). Explants from position a (terminal tips) produced most buds, but means were not significantly greater than the explants harvested from other positions (Table 28). No fungal or bacterial contamination was observed.

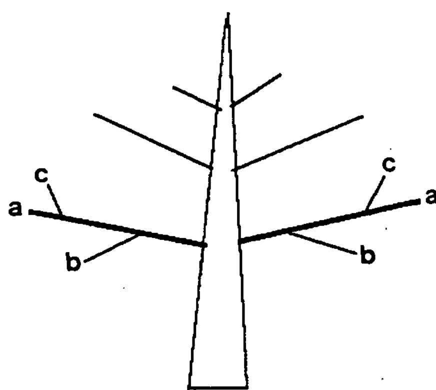


Fig. 6 : Diagram of the sampled apical meristems' gradients.

Table 28 : Effects of the position of the explants on the mother plant on establishment of cultures from 50 year-old *P. vera* trees*.

Explant position**	Survival (%)	Average shoot length*** (mm) ± SE	Average bud no*** per explant ± SE
a	50	6.2 ± 0.8 a	2.7 ± 0.3 a
b	60	6.7 ± 0.7 a	2.3 ± 0.3 a
c	75	7.4 ± 0.6 a	2.6 ± 0.3 a

**Indicates that figures presented are the means of 20 replicates observed on 35 day of culture. **Indicates that the lowercase letters represent the position of explants harvested in diagram of juvenility gradients in pistachio in Figure 6. ***Indicates that means in a column followed by the same lowercase letters are not statistically different at P = 0.05 level of significance according to the Student's t-test.*

After 35 d there was no further increase in shoot length in cultures, but shoots elongated with 2-3 pairs of rolled leaves or leafy structures. However, multiple shoot development was never observed adjacent to the axillary shoot when the surviving explants were subcultured or recultured onto a new medium. On all the types explants of tested considerable callusing was

observed at the base of the explants after a second subculture as observed in the previous experiments.

3.3.3 : Effects of time of the year (season) when explants were harvested

The time of the year when explants were harvested has been reported to influence their establishment and growth *in vitro* (Bonga and Alderson 1991). To test the influence of season on culture initiation, shoot tips were harvested in mid-June and mid-July 1994, and in early January and early March 1995. All explants were cultured using Method II as described in section 3.3.1, on MS medium supplemented with 1 mgl^{-1} BAP. Rates of survival of explants were assessed at the end of a 10-week incubation period.

Table 29 : Effects of time of the year explants were harvested.

Date	Total number of explants	% survival
Early-January 1994	30	27
Early-March 1994	30	54
Mid-June 1995	30	84
Mid-July 1995	30	77
χ^2 test (3 df)		P < 0.01

The results shown in Table 29 showed that the number of surviving mature explants was affected by the time of the year that the explants were harvested. The differences in the frequencies of explants surviving between the tested dates were highly significant. ($P < 0.01$, Table 29) with the highest percentage (84%) obtained from the mid-June 1994 harvested explants. Harvesting of explants in early January resulted in a significantly reduced frequency of survival (27%).

3.3.4 : Effects of cytokinins

The present experiment was carried out to investigate the effects of cytokinins on the establishment of meristem cultures from 50-year-old pistachio explants (Method II). A range of cytokinins such as BAP, K, 2iP, Zea and TDZ were added to the medium for culturing of meristems at 0.5, 1, 2, and 4 mgl^{-1}. The cytokinin BAP was also incorporated with 2iP (1 mgl^{-1}) to study their combined effect on the culture establishment. Shoots were collected from field grown *P. vera* trees in June 1994 shown to be the optimal date for explanting (section 3.3.3), and were kept in tap water for two days at room temperature in order to force the development of the axillary buds. Explants were cultured on agar-solidified MS medium in continuous light for

5 weeks and they were subcultured on fresh MS medium containing half of the growth regulator used for initiation treatments and incubated under standard conditions. The average shoot length and number of buds per explant was assessed after 5 weeks of culture. A linear logistic regression analysis was used to analyse the probability of obtaining surviving explants after 35 days of culture and a chi-square analysis was used to analyse the calli producing explants after 70 days of culture.

Table 30 : Effects of cytokinins on the establishment of meristem tips of 50-year-old *P. vera* trees*.

Hormonal treatments (mgl^{-1})	The average shoot length (mm)* ± SE	The average number of buds* per explant ± SE	Percentage (%) of the number of the explants producing callus**
1: BAP (0.5)	7.7 ± 0.4	4.3 ± 0.3	55
2: BAP (1.0)	8.2 ± 0.7	3.5 ± 0.3	60
3. BAP (2.0)	7.9 ± 0.4	4.8 ± 0.3	60
4: BAP (4.0)	2.4 ± 0.2	1.8 ± 0.2	75
5. BAP (0.5) ± 2iP (1.0)	6.0 ± 0.4	4.2 ± 0.3	65
6. BAP (1.0) ± 2iP (1.0)	4.7 ± 0.4	3.7 ± 0.3	70
7. BAP (2.0) ± 2iP (1.0)	3.6 ± 0.4	3.8 ± 0.3	75
8. BAP (4.0) ± 2iP (1.0)	3.1 ± 0.5	2.7 ± 0.2	76
9. Kin (0.5)	2.6 ± 0.2	2.4 ± 0.2	60
10. Kin (1.0)	2.9 ± 0.3	3.2 ± 0.2	50
11. Kin (2.0)	3.1 ± 0.1	2.0 ± 0.1	65
12. Kin (4.0)	4.3 ± 0.2	1.4 ± 0.1	70
13. 2iP (0.5)	4.2 ± 0.3	3.0 ± 0.2	45
14. 2iP (1.0)	3.6 ± 0.2	3.0 ± 0.2	65
15: 2iP (2.0)	3.8 ± 0.2	3.2 ± 0.2	75
16: 2iP (4.0)	3.8 ± 0.3	2.2 ± 0.2	70
17: Zea (0.5)	4.6 ± 0.2	1.6 ± 0.1	75
18: Zea (1.0)	3.8 ± 0.2	1.6 ± 0.1	65
19. Zea (2.0)	3.9 ± 0.3	1.6 ± 0.1	70
20. Zea (4.0)	3.2 ± 0.4	1.2 ± 0.1	75
21. TDZ (0.5)	4.6 ± 0.2	2.1 ± 0.2	50
22. TDZ (1.0)	3.1 ± 0.2	2.5 ± 0.1	65
23. TDZ (2.0)	3.6 ± 0.2	2.2 ± 0.2	75
24. TDZ (4.0)	3.3 ± 0.3	1.6 ± 0.1	75
F test (337df)	P < 0.001	P < 0.001	
χ^2 test (23 df)			P > 0.05

**Data recorded on the 35th day and represent an average of 20 replicates per treatment. **The percentage of the number of the explants producing callus after 70 days in culture from applied cytokinins and concentrations on the establishment of meristem tips of 50-year-old P. vera.*

Multiple shoot initiation was never observed in any of the hormonal treatments tested (Table 30) after 35 days in culture. The best multiple-shoot response was noticed in BAP (1.0 mgl^{-1}) (Table 30, Treatment 2) with an average shoot length of 8.26 ± 0.75 per explant on the 35 th day. 2iP (1.0 mgl^{-1}) and BAP (0.5, 1.0, 2.0 and 4.0 mgl^{-1}) in combination did not improve elongation of shoots. None of other tested cytokinins induced good response in shoot elongation.

After 35 d there was no further increase in shoot length in the treatments tested, but shoots elongated with 1-2 pairs of leaves. Significant differences were observed in mean bud number among the five cytokinins tested at different concentrations ($P < 0.001$, Table 30). Explants on BAP produced the most buds. Although more than two buds were produced per explants on TDZ, Zea and K, the multiplication rate was significantly lower than with BAP. The percentages of the explants producing callus are also presented in Table 30. There was no evidence of a significant difference in the frequencies of explants producing callus between the results obtained from different cytokinin treatments ($P > 0.05$, Table 30).

Table 31a : Analysis of deviance of the surviving explant from the applied cytokinins and concentrations on the establishment of meristem tips of 50-year-old *P. vera* trees after 5 weeks of culture.

Change	df	Deviance	Mean deviance	Ratio
+ con.	3	24.20	8.069	7.41
+ cyt.	5	12.27	2.045	1.30
Residual	15	10.24	0.68	
Total	23	46.71		

Table 31b : Fitted probabilities (*P*) of the surviving explants from the applied cytokinins and concentrations on the establishment of meristem tips of 50-year-old *P. vera* trees after 5 weeks of culture.

PGR Con. (mgl^{-1})	0.5	1.0	2.0	4.0
Cytokinin	*P*	*P*	*P*	*P*
BAP	0.82	0.78	0.78	0.57
BAP + 2iP	0.66	0.61	0.61	0.36
K	0.83	0.80	0.80	0.59
2iP	0.85	0.82	0.82	0.62
Zea	0.76	0.73	0.73	0.49
TDZ	0.80	0.76	0.76	0.53

The main effects of cytokinins and their concentrations were highly significant and significant ($P < 0.01$ and $P < 0.05$, respectively, Table 31a) for the survival of explants. The probability of the surviving explants remained relatively stable for the cytokinin concentrations, 0.5, 1.0 and 2.0 mgl^{-1} and was lowest for 4 mgl^{-1}. There was an obvious pattern between the cytokinin concentrations. For instance, 0.82 of explants survived after 35 days on the initiation medium with 0.5 mgl^{-1} BAP, but lower survival rates (0.78, 0.8 and 0.57, respectively) were observed with the concentrations of 1.0, 2.0 and 4.0 mgl^{-1} BAP. As can be seen from Table 31b there is an obvious pattern in the probability by the cytokinins tested for the culture initiation. The highest

probability of (0.85) was obtained with 0.5 mgl^{-1} 2iP while the lowest one (0.35) with 4 mgl^{-1} BAP and 1 mgl^{-1} 2iP (Table 31b).

When meristem tips were subcultured on to the same medium, gradual proliferation was seen over time, and the number of unhealthy shoots maintained a constant value during the five successive subcultures. Further subculturing in the same medium, but with half strength growth regulator treatments demonstrated that during 5 months of repeated subcultures the number of surviving explants on all cytokinin-supplemented medium gradually declined. After three subcultures on half-strength medium, some surviving explants were recultured on a growth regulator-free medium. Most of the explants produced translucent, rolled leaves. Shoot elongation was promoted under all treatments, but none of the buds showed bud breaking.

3.3.5 : Discussion

Several improvements were determined for multiplication of explants of mature pistachio. In order to encourage shoot elongation and axillary shoot formation, the explant type used for establishment, the position of explants on mother plants, the time of the year explants were harvested and influences of growth regulators were tested.

The establishment of explants was found to be significantly affected by the method used for culture initiation with 40 and 10% contamination, and 60 and 90% survival being obtained using the first and second methods, respectively. These results for culture initiation are not in agreement with those obtained by Abousalim (1990) who found that a high survival (85%) rate was obtained by using axillary buds either non-forced or forced to leaf out as bud cultured system, or longer explants (i.e. nodal and apical segments) as shoot culture system. It should be noted that in agreement with the results reported by Barghchi (1985) establishment of nodal segments or apical tips (using Method I) in culture was very difficult due to severe browning and high contamination when Method I was used for the culture initiation. The origins of explants on mother plants did not influence their establishment and growth. Explants harvested from terminal, basal and an intermediate position of a branch gave relatively stable survival rates (50, 60 and 75%, respectively). These results are in contrast to Abousalim's observation for mature pistachio explants harvested in mid-July that a significant increase in surviving explants was obtained using basal as compared with terminal explants.

The maximum percentage of surviving explants was obtained from explants harvested in early-June. There was evidence of a significant difference in the frequencies of surviving explants between the results obtained from explants harvested at the different dates. However, it should be noted that difficulties in securing permanent supplies of adult materials did not allow a

continuation of investigation on the establishment of such materials throughout a complete year. Besides, these results are in agreement with other results reported in pistachio (Barghchi 1985). In addition experiment 3.3.3 shows that an empirical study is necessary so that its effects should be determined for further experiments.

The maximum shoot length and highest bud number were obtained by using the cytokinin BAP. The combination of BAP with 2iP (1 mgl^{-1}) did not influence the effectiveness of BAP. Callus production occurred in all treatments with a substantial increase by increasing the growth regulator in callus development on developing shoots when media were supplemented with any of the cytokinins (Table 30). These results are in agreement with those obtained by Abousalim (1990) who found that callus production was evident in all BAP treatments tested (0.0, 0.5, 1.0, 2.0 and 4 mgl^{-1}) The results presented in section 3.1.3. are the first promising results for the establishment of mature pistachio cultures. Before this study none of the other researchers reported establishment of proliferating shoots. However, future work will confirm if the effect of the time of the year explants were harvested was studied throughout a year for the establishment of rapidly proliferating shoot cultures.

3.4 : REGENERATION AND ESTABLISHMENT OF PLANTLETS

In this part, the results of studies into the formation of plantlets using axillary derived shoots from seedlings of *P. vera* are presented. Nodal segments containing 1 or 2 axillary buds were collected from 6-month-old greenhouse-grown seedlings. The explants were sterilised as explained in section 3.1.9. In a series of experiments, the efficiency of the methods developed for future cloning and vegetative propagation of this species was examined in relation to the rate and quality of the adventitious root systems induced and corresponding growth of the shoots. The explants were pre-treated for 10 days in a mixture of 1 + 2 + 0.05 mgl^{-1} NAA + IBA + BAP, respectively before being transferred to the root induction medium. For the rooting treatments, the pre-treated shoots were incubated either in 24 mm test tubes (one explant per tube) (Fisons) containing 10 ml full strength of MS medium, or 400 ml Magenta 7 vessel (4 explants per vessel) containing 50 ml full strength of MS medium supplemented with 0.3% sucrose and 0.7% agar, after it had been supplemented by different combinations of growth substances. For root development, once root primordia became visible, the treated shoots were transferred to 400 ml plastic Magenta 7 (4 explants per vessel) vessels containing 50 ml of medium but devoid of growth substances.

3.4.1 : Application of growth substances

3.4.1.1 : Effects of auxins and auxin combinations (IBA +NAA) on rooting of *in vitro* shoot

In vitro rooting of *P. vera* has been achieved for shoot cultures derived from adolescent seedling material (Barghchi and Alderson 1989, Bustamante-Garcia 1984, Abousalim 1990), and shoot cultures derived from one-to four-year-old grafted plants (Martinelli 1988; Abousalim 1990). In the present study, axillary shoots (1.5-2 cm in height) were excised after 2 subcultures on multiplication medium containing 3 mgl^{-1} BAP + 1 mgl^{-1} 2iP. They were cut diagonally to expose the base of the stem. 10 days after pre-treatment, the explants were then subjected to media containing various combinations of two auxins as follows:
IBA at concentration of 0, 1, 2, and 4 mgl^{-1}
NAA at concentration of 0, 1, 2, and 4 mgl^{-1}.
The explants were transferred to auxin-free medium once the root primordia were visible. A final reading was taken after 6 weeks in which the number of roots and the percentage of the rooted shoots were recorded.

The cultured shoots exhibited various rooting responses according to the IBA/NAA treatments applied (Fig. 7). No rooting was ever observed when hormone-free medium was used. There were significant differences in the frequencies of rooting shoots between treatments ($P < 0.05$, Fig. 7). With increasing IBA or NAA concentration (up to 4 mgl^{-1}) the frequency of shoots forming roots did not increase significantly when the results were compared without the control. IBA alone was slightly more effective in root induction than NAA. A concentration of 4 mgl^{-1} of IBA or NAA did not improve the percentage of rooting. The percentage of shoots forming roots was sharply enhanced with the mixture combination of IBA and NAA. The highest percentage of rooting (60%) was obtained with a combination of 1 mgl^{-1} IBA + 2 mgl^{-1} NAA. However, in the presence of NAA the percentage of shoots which formed roots was decreased at IBA concentrations of 2 and 4 mgl^{-1}. Mixture of two auxins with intermediate concentration (NAA + IBA) promoted rooting more effectively than any one auxin.

After 6 weeks of culture, significant differences in root number per shoot were observed between the treatments applied. (Fig. 8). Optimal mean root numbers per shoot were obtained on media supplemented with 2 mgl^{-1} IBA and 1 mgl^{-1} NAA. However, there was no indication of an increased rate of root number, whereas no significant differences in root number were observed when the results obtained with one auxin were compared between treatments (Fig. 8).

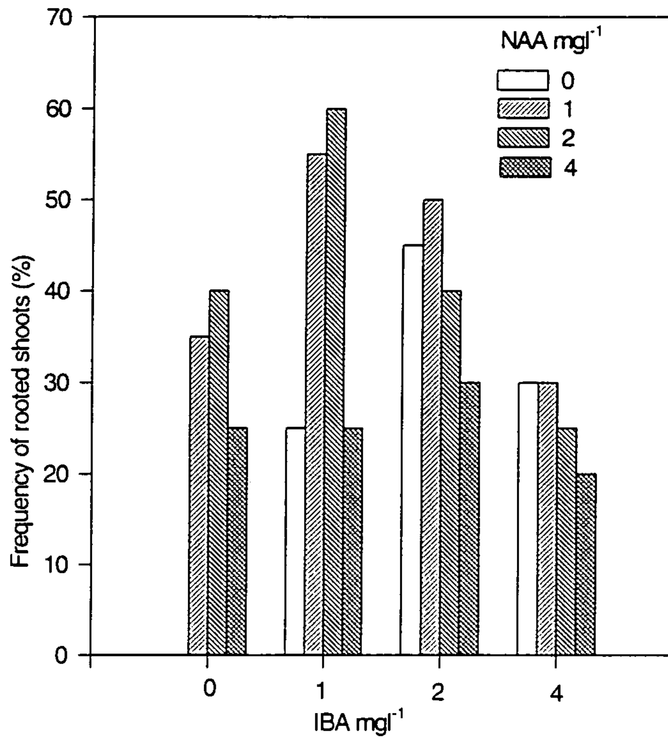


Fig. 7 : Effects of IBA and NAA on the frequency of root induction on nodal bud segments of P. vera. 20 shoots subcultured twice were used for each combination. χ^2 test (15 df): $P < 0.01$.

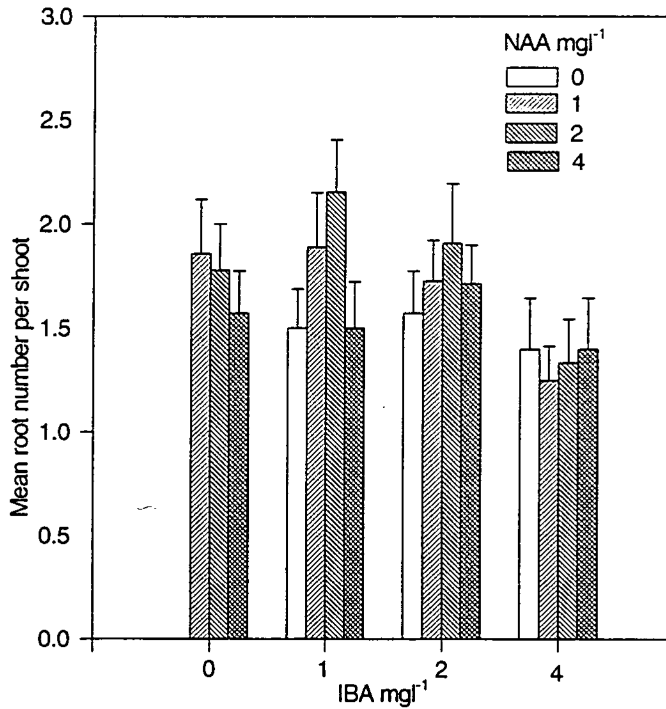


Fig. 8 : Effects of IBA and NAA on mean root number of P. vera nodal bud segments after 6 weeks of culture. Vertical bars represent the standard error of the mean. 20 shoots were used for each combination.

3.4.1.2 : Application of different concentrations of the cytokinin BAP

Effect of auxin (IBA) and cytokinin (BAP) combinations on *in vitro* rooting of shoot was also tested. After pre-treatment, the explants were transferred to Magenta 7 vessel (four explants per vessel) containing 50 ml agar-solidified (0.7%, pH 5.7) MS medium supplemented with various combinations of IBA and BAP as follows:

IBA at concentration of 0, 1, 2, and 4 mgl^{-1}

BAP at concentration of 0, 0.05 and 0.5 mgl^{-1}.

Each treatment was replicated with 20 replicates. The tubes were placed in the incubators at random. Results recorded after 6 weeks of culture included the number of roots and the percentage of the rooted shoots. After shoots had been treated as in the previous experiment until the first emergence of roots, they were transferred onto media devoid of growth substances for a total incubation time of 6 weeks, where frequency of rooting, and root numbers per plant were evaluated.

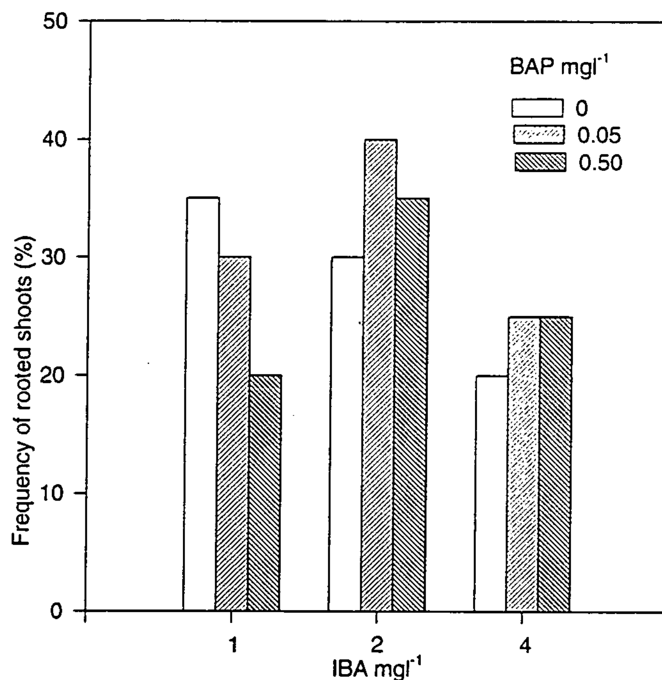


Fig. 9 : Effects of IBA and BAP on the frequency of root induction on nodal bud segment of P. vera. 20 micro shoots subcultured twice were used for each combination. χ^2 test (8 df): $P < 0.01$.

The cultured shoots exhibited various rooting responses according to the IBA/BAP treatments applied (Fig. 9). In the presence of BAP at a low level (0.05 mgl^{-1}) the percentage of shoots forming roots was enhanced with IBA concentration of 2 mgl^{-1}. However, in the presence of BAP at a high level (0.5 mgl^{-1}) the percentage of shoots forming roots declined. In addition the inclusion of BAP was also inhibitory to the growth of roots with all IBA concentrations tested when the results were compared with the mixture of IBA/NAA. Concerning root number, the

result presented provide evidence of a negative effect of the combination of BAP and IBA (Fig. 10) the 2 mgl^{-1} IBA and 0.05 mgl^{-1} BAP treatment produced optimal root number per shoot.

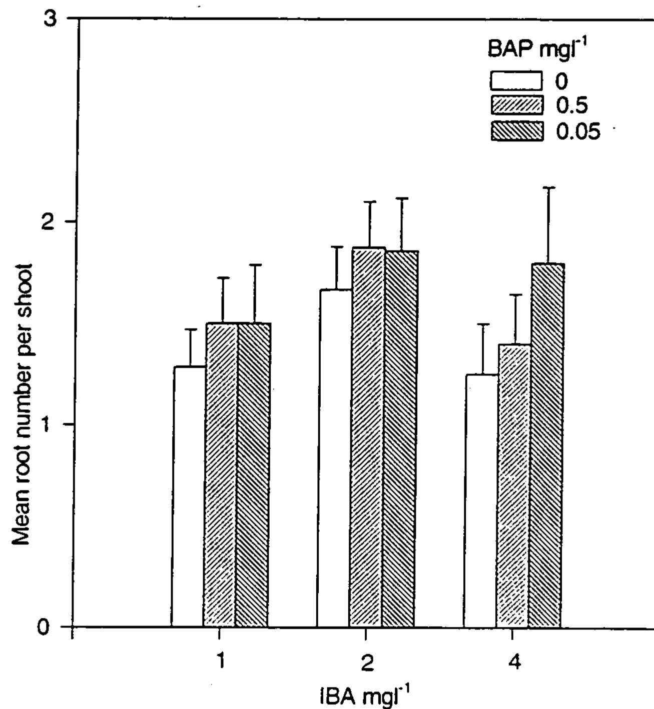


Fig. 10 : The effect of IBA and BAP on mean root number of P. vera nodal segments after six weeks of culture. Vertical bars represent the standard error of the mean. 20 shoots were used for each combination.

3.4.2 : Effects of subculture number on rooting of shoots *in vitro*

In this experiment, the effect of subculture number on rooting was investigated using *P. vera* seedling materials. Nodal segments were excised from *in vitro* grown seedlings as well as from materials obtained from shoots after one, five and ten subcultures on multiplication medium containing 3 mgl^{-1} BAP and 1 mgl^{-1} 2iP. After shoots had been pre-treated as above for 10 days, they then were transferred onto 50 ml of media supplemented with 1 mgl^{-1} NAA, 1 mgl^{-1} IBA, 3% sucrose and 0.7% agar for a total incubation of 6 weeks, where the frequency of rooting and root number per plant were evaluated. The shoots were transferred on auxin-free medium once the root primordia were observed.

Shoots subcultured 10 times responded better than did those which had been subcultured once and five times on MS multiplication medium. The highest percentage of rooting (55%) was obtained from 10 times cultured material. However, there was not a significant difference in the frequencies of the rooted shoots between the treatments ($P > 0.05$). More than 50% of the explants produced callus on once subcultured explants.

Concerning the root number, again shoots subcultured 10 times produced the optimal mean root number per shoot (Table 32). However, no significant difference existed between treatments in respect to root number.

Table 32 : Effects of subculture number on root number and percentage of rooting of *P. vera* seedlings derived micro shoots (n = 20).

	No. of subculture			F test	χ^2 test
	1.0	5.0	10	(23 df)	(2 df)
Mean root number per shoot	1.5	1.6	2.0	N.S.	
% rooting	20	45	55		N.S.

N.S.: Indicates no significant difference.

3.4.3 : Clonal effects of rooting shoots

Attempts in previous experiments to induce rooting of pistachio with sufficient rate using nodal segments explanted from a mixed seedling population had proved disappointing. The next experiment was to determine if conditions may be improved so that higher rooting percentages could be obtained. In this experiment, explants that had been cloned from three different rooted plantlets were utilised. In this way, uniform explant populations could be made available. Explants having 2 axillary buds were used. They were pre-treated for ten days as mentioned before (section 3.4) and then cultured in an MS medium containing 1 mgl^{-1} IBA, 1 mgl^{-1} NAA, 3% sucrose and 0.7% agar. The explants were incubated in continuous light at 25°C for a period of 6 weeks. Once root primordia became visible, they were then transferred to the same basal medium without auxins.

Table 33 : Clonal effects of rooting shoots on the percentage of rooted explants of *P. vera* (n = 20).

	No of clone			χ^2 test (2 df)
	1	2	3	
% of rooting	90	85	55	P < 0.05

The cultured shoots exhibited various rooting responses according to the clones tested (Table 33). There was some evidence of a significant difference in the rooting percentages obtained between the clones ($P < 0.05$, Table 33). Six weeks after the explants were cultured in MS medium, the rooting had reached 90%, 85% and 55% in the clones 1, 2 and 3, respectively. The roots produced in each clone showed similar growth characteristics.

3.4.4 : Callus growth

Of the treatments tested, the production of callus at the bases of the treated shoots rooted was observed. A reduction of callus production was observed when more mature shoots were used for rooting. Containers (24 mm test tubes (one explant per tube) (Fisons) containing 10 ml full strength of MS medium) used for rooting also played an important role for the production of callus at the stem of the treated shoots.

3.4.5 : Acclimatisation

Attempts to acclimatise tissue culture derived plantlets were performed in a growth room with light intensity of 20 μmol m^{-2} s^{-1}. *In vitro* rooted shoots, after 6 weeks on root-induction media at a photon flux density of 40 μmol m^{-2} s^{-1}, were washed overnight under running water and they were then potted in a mixture of sand and peat (granulated sphagnum moss peat, pH 5.5) (1:1, v/v) and watered every 3 days with Solinure 7 solution (Fisons). Plantlets were covered with a Pyrex beaker to maintain 90±5% relative humidity for 4 weeks before transfer into greenhouse conditions (25°C day; 20°C night; 18-h day length). Acclimatisation was achieved in two stages: the first was maintained at 90±5% RH for 2 weeks and the second at 70±5% RH for 2 weeks before plantlets were transferred to normal greenhouse conditions.

The plantlets started to elongate and form new leaves within two weeks after transfer to the growth room. The survival of plantlets was higher when they were covered with a glass in the growth room. A two-week period of gradual lowering of humidity from 90% to 70% had an effect upon the plantlets. Shoot growth resumed at week four and there were at least two new leaves on each plant. Fungal contamination of the substrate and plantlet was occasionally observed. Slight opening of the glasses during acclimatisation prevented rapid dehydration of the aerial parts and allowed a gradual and progressive acclimatisation of the plantlets. After 4 weeks from transplanting propagules to compost mixture in a growth room, well-established shoots were transferred into a greenhouse.

3.4.6 : Establishment of plantlets

A final assessment of the effectiveness of any *in vitro* micropropagation technique must be delayed until information becomes available on the performance *in vitro* of the plantlets produced. Accordingly, morphological observations were directed to obtaining the patterns of growth and development of adventive and axillary plantlets, and to comparing them to seedlings of pistachio one year after transfer into soil.

After a year in soil, both seedling and micropropagated plants showed similar growth characteristics under the same greenhouse conditions. In terms of shoot growth, both seedling and micropropagated plantlets exceeded that of very little axillary shoots. The root characteristics of the micropropagated plantlets were also very similar to those of the seedlings. A couple of months after transplanting the majority of plantlets produced very deep root systems. Once the root reached the bottom of the containers, the growth of the plantlets was gradually reduced. Frequent transplanting of the plantlet into a new container also affected the growth characteristics. These preliminary observations showed that because of the unnatural conditions, the patterns of growth and development of micropropagated plantlets and seedlings of pistachio can not be determined under greenhouse conditions.

3.4.7 : Survival and performance of plantlets *in vivo*

The method developed for plantlet acclimatisation was not satisfactory. In the first place a high percentage of plantlet survival (80%) in the greenhouse was obtained but a low percentage of plantlets (less than 50%) resumed their growth after 1 year. Not only the regenerated plantlets *in vitro* but also seedlings raised *in vivo* died a couple of months after transfer to soil. This could be a lack of mycorrhizal formation on this rooting system that had to be induced. Plantlets that had been propagated using optimal conditions outlined earlier in this study, displayed serious slow growth of shoots or some fast and deep growth of roots. These are other areas which need to be studied in the future.

3.4.8 : Discussion

The initiation of roots was found to be specially dependent upon auxin. In the present study seedling material of *P. vera* was readily rooted *in vitro* in the presence of auxin. This is in accordance with the usual findings that this hormone is a requirement for root induction (Barghchi 1982; Bustamante-Garcia 1984 and Abousalim 1990). The present data also indicate that the rooting response is significantly influenced by the applied concentrations. This finding is consistent with the results recorded by Bustamante-Garcia (1984) who found that in the liquid medium rooting reached 100% with 10 mgl^{-1} NAA or in the agar-solidified medium rooting was 100% with 10 mgl^{-1} IBA. However, these recorded rooting rates were not in agreement with the present study when the explants rising from a mixed seedling population were used (Fig. 7). Cultured shoots also responded according to the auxin level employed, a fact that has been reported by others (Barghchi 1982; Martinelli 1988; Abousalim 1990). In the present study, a mixture of two auxins (IBA+NAA) elicited maximum rooting (90%) using a cloned-stock, which is

not a fact that has been reported by other workers on pistachio (Fig. 7). However, a mixture of auxin-cytokinin inhibited rather than promoted adventitious rooting.

In the present study, a low rate of rooting (60%) was reported in the shoots excised from a mixed seedling population. However, improvements obtained in rooting percentages (from 60% to 90%) were produced when cloned shoots were used, and this finding has not previously been reported for pistachio. Therefore, this may indicate that not only growth substances but also some other factor may limit root induction, and this merits attention before any practicable application.

There was no evidence of a significant difference in the frequencies of rooted shoots between the results obtained from shoots excised after one, five and ten subcultures. These results are in contrast to those obtained by Abousalim (1990) who found that a shoot which had been subcultured only once showed best potential for rooting as compared with shoots obtained after two subcultures. It was also suggested that explants taken from subcultured more than once and multiplication medium with a high cytokinin concentration conditioned insufficiently to *in vitro* conditions may have an effect on the conditioning of explants (Abousalim 1990).

Alternative approaches for rooting of pistachio under *in vitro* conditions have also been reported. Rooting of four-year-old *P. vera* material was achieved using the quick auxin-dip approach (Abousalim 1990). Up to 50% of the plantlets rooted with a mean of three roots. Per rooted shoot after five weeks of culture, 85% rooting was obtained on mature softwood cuttings of *P. vera* under mist but only after treatment with an extremely high level of IBA (35000 mgl^{-1}) (Al Barazi and Schwabe 1982). In the most recent study there is also a trend towards *extra vitrum* (*in vivo*) rooting procedures for seedling material (Abousalim 1990). Rooting was induced at all tested IBA levels with 100% rooting being obtained at 500 and 1000 mgl^{-1}. However, it should be noted that these results are in contrast to my preliminary observations for mature material (50-year-old) that none of the explants (100 cuttings) induced roots *in vivo* even with the application of high concentrations of IBA (data not presented). Data from this discussion demonstrate that *P. vera* seedling materials could be rooted *in vitro* with the combination of two auxins: the level of the combination appeared to be an important influence on the rooting response obtained.

CHAPTER FOUR

EMBRYOGENESIS STUDIES

CHAPTER FOUR : EMBRYOGENESIS STUDIES

As the objective of this part of this thesis was to study and compare the various aspects of *in vitro* embryogenesis in pistachio, tissue culture protocols and experimental systems for the regular induction and production of somatic embryos were an essential prerequisite. In this part, a series of experiments was carried out in order to develop methods for the establishment of embryogenic mass, and development of somatic embryos i.e. maturation, germination, plantlet development and plantlet acclimatisation using explants from immature fruits, juvenile and mature leaf explants, and zygotic embryos.

Section 4.1 comprises a major part of the investigations into the development and exploration of an embryogenic system *in vitro* culture, with the emphasis on the initiation and development of somatic embryos in immature fruit cultures. The objective of these experiments was to formulate a protocol of basic cultural conditions for the establishment of EMS and the development of an embryogenic system for the production of somatic embryos. Accordingly, screening experiments aimed to determine the effects of factors such as type, concentration and mode of application of exogenous growth substances, as well as tests on the nutrient medium in liquid or solid form were undertaken. Assessment of the nutritional requirement of cultured explants were simplified by considering separately the cytokinins and auxins. In sub-section 4.1.1, a series of experiments is described on the effects of the explants harvesting date of the induction of EMS. The morphological characteristics of the initiated calluses are examined in sub-section 4.1.2. In sub-section 4.1.3, a series of experiments are described concerning the maintenance of embryogenic mass and the establishment of suspension cultures. Sub-section 4.1.4 includes experiments that show the effects of sucrose (I), glucose (II), fructose (III) and lactose (IV). Subsection 4.1.5 deals with experiments on the effects of different basal salts formulations, i.e. Murashige and Skoog (MS), Woody plant medium (WP), Schenk and Hildebrandt (SH), and Gamborg's B-5 (G-5). Sub-section 4.1.6 deals with experiments on the effects of light intensity on dry matter and fresh weight of EMS. In sub-section 4.1.7, a series of experiments are described concerning the maturation of SEs. *In vitro* development and the morphological characteristics of zygotic embryos are examined in 4.1.8. In sub-section 4.1.9, a series of experiments is presented in which the effects of growth substances and sucrose on germination of SEs were investigated.

Section 4.2 presents the results from studies on the initiation of embryogenic callus, maturation and development of somatic embryos using leaf explants of pistachio, which laid the foundation for the subsequent work on the induction of somatic embryogenesis in regenerated leaf cultures of mature pistachio trees.

Finally, section 4.3 presents the results from studies on the initiation of embryogenic callus, maturation and development of somatic embryos using mature zygotic embryos of pistachio, which laid the foundation for the subsequent work on the induction of somatic embryogenesis in different pistachio genotypes.

4.1 : SOMATIC EMBRYOGENESIS IN CULTURED KERNELS OF PISTACHIO, *PISTACIA VERA* L.

In this section, a series of experiments was conducted to investigate the effects of various factors involved in the process of somatic embryogenesis using immature fruits of pistachio.

4.1.1 : Influence of date of explanting on the induction of embryogenic mass (EMS)

Immature fruits were collected between mid-June and early-August 1993, and mid-July and mid-September 1994 from a pistachio nut tree growing on the Ceylan-pinari state production farm, in the Urfa province of South-east Turkey. Fruits were transported to Edinburgh, by aircraft, where they were kept in a plastic bag. The fruits were stored in paper bags at 4°C prior to dissecting the kernels. The extracted kernels were surface sterilised as described in section 3.1.8. The kernels were cultured on two different basal media: WPM containing 500 mgl^{-1} casein hydrolysate and 50 mgl^{-1} l-ascorbic acid, and MS medium containing 500 mgl^{-1} casein hydrolysate and 50 mgl^{-1} l-ascorbic acid. The above media were also supplemented with various combinations and concentrations of the auxins NAA and 2,4-D and cytokinins BAP and TDZ at concentrations of 1, 2, and 4 mg l^{-1} for NAA and 2,4-D, and 1, 2, 4 and 8 mg l^{-1} for BAP and TDZ. Combinations of 2,4-D and NAA at 1 to 4 mg l^{-1} with BAP at 1 mgl^{-1}, and agar-solidified with 0.7% Difco Bacto-agar (Sigma Agar A1296), and treatments with BAP and TDZ were also tested in both MS and WP liquid media. Usually, the media were supplemented with 3% sucrose and the pH was adjusted to pH 5.7 prior to autoclaving at 121°C, 20P for 16 min. Four or five kernels were cultured in each Petri dish (90 x 20 mm) on 25 ml of induction medium, and incubated in continuous light at 25°C. Each treatment was initiated with 10 or 11 seeds. The presence of EMS was determined by morphological observations and histological investigations.

Initiation of EMSes was observed only in the kernels cultured on BAP supplemented liquid MS medium in early-August 1993, and mid-July and mid-September 1994 harvested fruits. EMSes were never induced by WP medium nor on solid medium. Therefore, these results will not count in the analysis. The morphological observations recorded for the rest of the tested treatments for the initiation of EMSes will be followed hereafter for all tested dates. After 4 weeks, both the

EMSes and the callus or callusing tissues could be distinguished on the different treatments. The EMSes developed only from liquid MS medium supplemented with BAP (1-8 mg l^{-1})

The initiation of EMS was significantly influenced by the fruit collection date (Table 34). There was evidence of significant differences in the frequencies of kernels induced EMSes between the tested dates on liquid MS media ($P < 0.05$, Table 34). The frequencies of EMSes (20%, 10% and 12.5%, respectively) were obtained from the fruits collected on July 15th, August 7th and September 10th.

Table 34 : Effects of fruit collection dates on initiation of embryogenic mass (EMS) in liquid MS medium from kernel cultures of pistachio in liquid medium*.

Collection data	Media	No. of fruits cultured		% of kernels induced
		Cultured	with EMS	embryogenic mass(EMS)
June 15th 1993	MS	44	0	0.0
August 7th 1993	MS	40	4	10
July 15th 1994	MS	40	8	20
September 10th 1994	MS	40	5	12.5
χ^2 test				$P < 0.05$

**Data show only the cytokinin BAP treatments.*

Among several PGRs and combinations tested, only the BAP treatments in liquid MS medium initiated friable embryogenic tissue, depending on the concentration of BAP in the induction medium. The first stages of the development of EMS visible on the external part of the explants were never observed before 4 weeks in culture. The explants cultured at the highest concentration of BAP (8 mg l^{-1}) did not induce EMS and formed degenerate black tissue. Two embryogenic lines were established from the fruits cultured on August 7, and the more friable one was chosen for further studies. The establishment of a friable embryogenic line is given in subsection 4.1.3 Somatic embryos were also produced from EMSes initiated in 1994 but they were not investigated in detail.

4.1.2 : Morphological observations on callus production in the agar-solidified MS medium

I) June 15 1993

On June 15th, immature fruits (2-4 mm in length) were in the early stage of fruit development where kernels could not be removed from the suspensor and testa. In fact, a preliminary experiment showed that the immature fruits harvested on June 15th may be cultured devoid of surface sterilisation because when the kernels were carefully removed with sterile forceps and

scalpel, and cultured on the induction medium they did not show any contamination. Moreover, the surface sterilisation method was applied to the intact fruits but not to the extracted kernels because all explants were oversterilised when the sterilisation method was applied as explained in section 3.1.8. Therefore, only 10 min NaOCl treatment was sufficient for sterilisation.

During the first two weeks on agar-solidified medium, the outer epidermal cells of all cultured intact immature fruits became a black-brown circle. Within the four weeks of explanting, all chopped fruits turned dark brown and gradually dehydration of the medium was also observed. Four weeks after incubation, the majority of the explants increased in size and were recultured onto a fresh medium defined as the induction medium. During the second and third subculturing or reculturing, the explanted fruits increased in size macroscopically but did not produce visible callusing tissues. Some incubated fruits (seeds) became black-brown and some increased a few times in size three months after the culture. Two weeks after the third reculturing, some explants revealed callusing tissues. Among the applied treatments, there was little variation in the size increase of the explants cultured to initiate embryogenic callus. Among the applied treatments in liquid medium, there was little variation in the size increase of the explants cultured to initiate embryogenic callus. In conclusion, embryonic callus was not established from the immature fruits harvested approximately 8 weeks after post-pollination. From the experiment, it can also be concluded that the developmental stage of the explanted material is important and it might be that the time of harvesting the explant influencing the requirements for initiating embryonic callus. The results obtained in this preliminary study may be used to develop further experiments with the aim of improving the factors relating to embryogenesis and the regeneration capacity of these plant tissues in culture.

II) August 7 1993

On August 7th, the fruits (10 mm in length, at the middle stage of ripening) were as big as the mature ones. The kernels extracted whose shell had been removed were surface sterilised as explained in section 3.1.8. After 4 weeks of culture, the growth responses of the cultured immature fruits of *P. vera* to the different plant growth regulators are indicated in Table 35. No callus initiation was observed on plant growth medium devoid of plant growth regulators.

In general, all explants on the different composition of growth regulators formed callus after 4 weeks of culture. In the initial stage of culture in the agar-solidified medium, the first sign of the activity started within 5 days but visible callus formation and growth from the intact fruits was observed within 14 days of the explanting. The initial callus in all treatments was white and friable and started to form from radicle sections of the initiated immature fruits. The presence of 2,4-D was considerably more effective than NAA, BAP and TDZ for stimulating callus initiation

and growth. The addition of BAP was observed to promote callogenesis. Auxins when incorporated into the medium supplemented with 1 mgl^{-1} BAP, 2,4-D slightly enhanced the friability of callusing tissues. The explant incubated on the BAP and TDZ treatments sprouted within 10 days as mature seeds but after three weeks of culture, most explants induced macroscopically visible callus formation. Calli produced in BAP treatments were fairly uniform, hard and green in colour with some red pigmentation. Three weeks after culturing, the calli produced in all other treatments ranged from white to greyish or yellow in colour.

Table 35 : Effect of growth regulators on callus induction in the agar-solidified medium four weeks after initiation.

Composition of growth regulators (mgl^{-1})						
2,4-D	NAA	BAP	TDZ	% Response	Quantity*	Remarks
–	–	–	–	100	–	germinated seeds
1				100	++	white callusing tissues
2				100	+++	white callusing tissues
4				80	++	white callusing tissues
1		1		90	++	white-greyish callusing tissues
2		1		100	++	white-greyish callusing tissues
4		1		90	++	white callusing tissues
	1	1		90	++	greyish callusing tissues
	2	1		80	+++	creamy callusing tissues
	4	1		80	++	creamy callusing tissues
	1			80	++	creamy callusing tissues
	2			100	++	creamy callusing tissues
	4			100	++	creamy callusing tissues
		1		88	+	green callusing tissues
		2		100	++	greyish white callus
		4		91	++	deep green callusing tissues
		8		91	++	deep green callusing tissues
			1	100	+	green callusing tissues
			2	88	++	reddish-green callusing tissues
			4	100	++	reddish callusing tissues
			8	91	++	reddish callusing tissues

**Indicates that are - absent, + poor, ++ fair, +++ good, ++++ very good.*
Note: Results are from a single experiment consisting of 10 or 11 explants per treatment.

Regardless of the cytokinin used, immature fruit callusing tissues became green in colour after three weeks. Rapidly growing calli were initiated from immature fruits on all medium formulations containing 2,4-D and NAA, where very little callusing tissue was produced on a medium containing only BAP and TDZ alone. In general, middle level concentrations of tested auxins and cytokinins in the callus-induction medium favoured friable callusing tissues. In the light of my observations, the best callus inducing material is white-yellow and hard seeds. Four weeks after culturing, the intact fruits generally gave rise to two distinct types of calli : a) greyish yellow compact and nodular; b) nodular, translucent creamy yellow. When calli were subcultured on their original media for another four weeks, in general most of the callus browned and died, and

neither embryogenic structures nor friable maintained callus could be observed. However, when the callus from the initiating treatments with different concentrations of plant growth regulators was transferred to 1 mgl^{-1} BAP, calli were well maintained. This callus, which did not form EMS in the subculturing media was subcultured regularly for more than a year by monthly subculture onto MS medium.

III) July 15 and September 10 1994

On July 15, the immature fruits (7-10 mm in length) were at the beginning of fruit development. On September 10, the fruits were fully ripe. Surface sterilisation of the fruits on both dates was achieved using the standard surface sterilisation technique as explained in section 3.1.8. Callus development and morphology on agar-solidified medium was similar to one recorded for August 7, 1993. Therefore, the development of calluses will not be repeated here.

4.1.3 : The initiation and maintenance of embryogenic masses and the establishment of suspension cultures of pistachio

Attempts to establish a fine cell suspension from clusters of EMSes resulted in failure. However, rapidly growing clusters of embryogenic masses were obtained. In the following series of experiments attempts were made to optimise growth in suspension cultures. Suspension cultures were established from pistachio kernels harvested on August 7 th 1993. The basal salts mixture (MS, WP, SH and G-5) and nine carbohydrates with or without 1 mgl^{-1} BAP were all examined. Suspension cultures were started from EMSes (0.5 g wet weight) using Erlenmeyer flasks of 250 ml capacity containing 50 ml of liquid MS medium without agar. The flasks were then sealed with a double layer of aluminium foil and placed on an orbital shaker at 98 rpm at a light intensity of 25 μmol. m^{-2} sec^{-1} photon flux density and a temperature of 25°C. They were subcultured every 10 or 12 days. Since the embryonic mass cultures consist of a highly vacuolated suspensor and densely cytoplasmic embryonic cells, the dry matter of these cultures is strictly correlated with the portion of embryonal cells (Schuller and Reuter 1993). Growth was determined by taking wet and dry weight measurement rather than by cell count data, because the cells are in very tight clusters, and the clusters are not completely digested with enzyme peroxidase and speed shaking. For fresh weight (FW) measurements the embryonic tissue was weighed in a plastic Petri dish, and was left at room temperature for at least a couple of hours in order to remove the liquid medium. Then total dry weight of the embryonic mass was obtained by putting the embryonic mass on a pre-weighed paper case previously stored in a desiccator in an oven at 80°C for at least 48 h. They were then weighed, after cooling in a desiccator, placed in the oven for another 24 h, cooled and weighed. If there was no decrease in weight for any of the treatments, it was assumed that they were oven dry. All flasks were sampled after 10 days of

culture, and the fresh weight and dry matter were determined as explained above. Each growth measurement represents the mean of 5 flasks per treatment. Each experiment was done at least twice. All the embryonic mass suspension experiments described in the subsequent figures were done with the same embryonic line (originating from a single fruit).

4.1.4 : Effects of carbohydrates on dry matter content and fresh weight in EMS cultures of pistachio

In order to study the influence of various carbohydrate sources on embryogenic mass proliferation, 5 times subcultured embryogenic mass was used in experiments with different carbohydrate concentrations on a MS medium modified by the addition of 2, 4, 6, 8, 10 and 12% of sucrose, glucose, lactose, fructose, ribose and xylose with or without 1 mgl^{-1} BAP. Only four tested sugars (sucrose, glucose, fructose and lactose) in various concentrations in the liquid MS medium had any pronounced effect on the dry matter (Fig. 11, 13, 15 and 17) and the fresh weight (Fig. 12, 14, 16 and 18). Therefore, the results obtained for other tested carbohydrates are not presented.

4.1.4.1 : Effect of sucrose on dry matter content and fresh weight in EMS cultures of pistachio

In this experiment, the effect of sucrose, an important media component, was studied. The effect of sucrose was tested in Erlenmeyer flasks containing 50 ml of MS medium supplemented with or without 1 mgl^{-1} BAP, and the following concentrations (w/v) of sucrose: 2%, 4%, 6%, 8%, 10% and 12% were tested.

The effects of sucrose concentrations on dry matter as % of the fresh weight are shown in Fig 11. Cultures grown on a growth regulator-free medium attained approximately 10% higher dry matter than cultures grown on a BAP treated medium. The statistical analysis of data showed that there were very highly significant differences in the dry matter content of EMSes between the tested treatments ($P < 0.001$). There was a steady increase in the dry matter content of the embryogenic mass on both media with BAP and growth regulator-free, and with an increase in the concentrations of sucrose up to concentration 10% of sucrose and a slight decrease thereafter (Fig. 11). In both with/without BAP treatments there was a strong positive correlation between dry weight and sucrose concentration ($r = 0.98$ and $r = 0.96$, respectively). However, there was a negative correlation (Fig. 12) between fresh weight and sucrose concentration ($r = -$ 92 and $r = -0.91$, respectively). The effects of sucrose concentrations on fresh weight are shown in Fig. 12. With increasing sucrose the fresh weight production declined (Fig. 12). In all the other treatments tested, the ratio of final to initial fresh weight of the embryonic tissue ranged from 3.5

to 8.9 on MS medium supplemented 10% and 4% sucrose, respectively. New embryonic tissue proliferated during the mid-level sucrose treatments was slightly friable with a deep green texture.

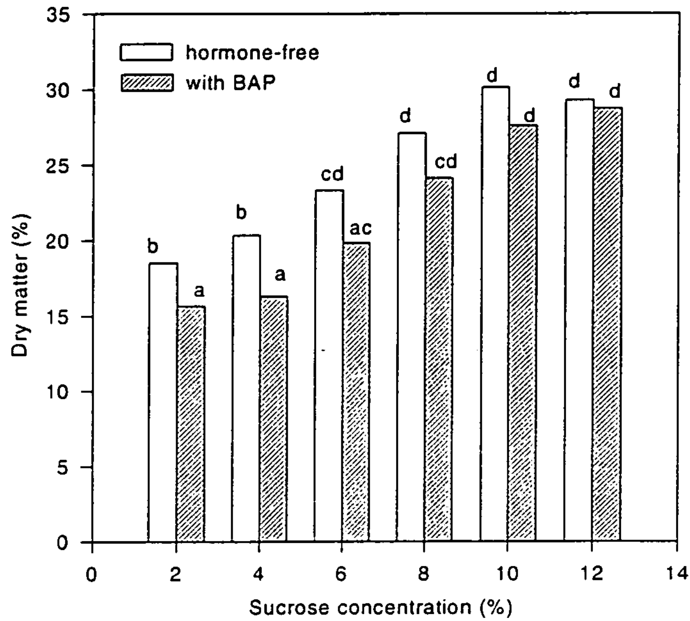


Fig. 11 : Relationship between sucrose concentration (%) and dry matter of EMS (%) on media supplemented with 1 mgl^{-1} BAP and on growth regulator-free media. Different lowercase letters above any two columns indicate that these two means are statistically different at p = 0.05 according to the Student t-test. The data for the mean of two experiments with 5 replicates. Embryonic tissues had been subcultured 5 times before the experiments performed.

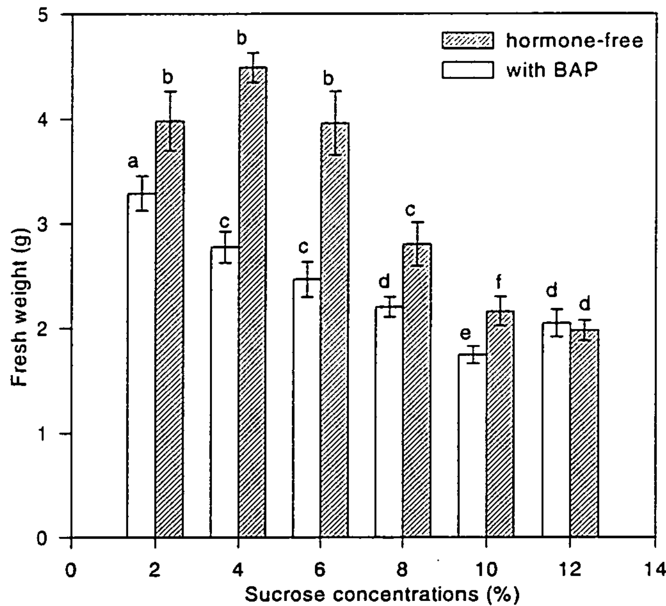


Fig. 12 : Relationship between sucrose concentration (%) and fresh weight of EMS (g) on media supplemented with 1 mgl^{-1} BAP and on growth regulator-free media. Different lowercase letters above any two columns indicate that these two means are statistically different at p = 0.05 according to the Student t-test. The data for the mean of two experiments with 5 replicates. Embryonic tissues had been subcultured 5 times before the experiments performed. Vertical bars represent the standard error of the mean.

In general, the embryonic tissues of all treatments proliferated with a fine texture and just visible immature somatic embryos after 10 days of growth. The results presented in Fig. 11 and 12 indicate that the EMSes showed the best growth response between 6% and 10% sucrose in terms of dry matter content, and between 2% and 6% sucrose in terms of fresh weight, respectively. Therefore, 6% of sucrose was now chosen to proliferate EMSes for subsequent studies.

4.1.4.2 : Effect of glucose on dry matter content and fresh weight in EMS cultures of pistachio

In this experiment, the effect of glucose was studied on proliferation of EMS. The effect of glucose was tested in Erlenmeyer flasks containing 50 ml of MS medium supplemented with or without 1 mgl^{-1} BAP, and with the following concentrations (w/v) of glucose: 2%, 4%, 6%, 8%, 10% and 12%.

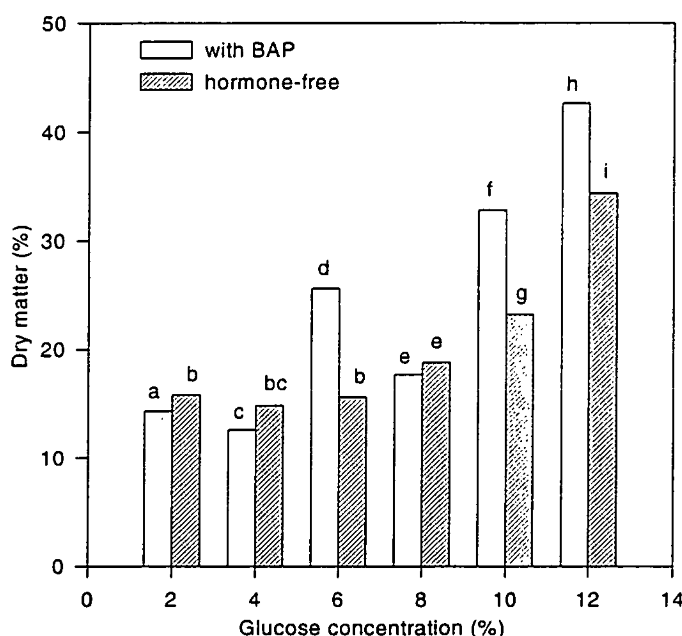


Fig. 13 : Relationship between glucose concentration (%) and dry matter of EMS (%) on media supplemented with 1 mgl^{-1} BAP and on hormone-free media. Different lowercase letters above any two columns indicate that these two means statistically different at p = 0.05 according to the Student t-test. The data for the mean of two experiments with 5 replicates. Embryonic tissues had been subcultured 5 times before the experiments performed.

The effects of glucose concentrations on dry matter are shown in Fig 13. Statistical analysis of data showed that there were very highly significant differences in the dry matter content of EMSes between the tested treatments ($P < 0.001$). In general, an increase in glucose concentrations increased the dry weight (Fig. 13). There was a steady increase in the dry matter content of the embryonic mass on both media with or without BAP, and with an increase in the concentrations of glucose up to a concentration of 12% of glucose. The EMSes treated with 12%

of glucose without BAP had a mean dry weight about four times higher than the initial one. An analysis of results showed that the fresh weight was also significantly influenced by the concentrations of glucose ($P < 0.001$, Fig. 14). The EMS treated with 4% glucose with BAP increased in fresh weight at a maximum of 11.3 after 12 days. The ratio of final to initial fresh weight of the embryonic tissue ranged from 2.92 to 11.3 on MS medium supplemented 12% and 4% glucose, respectively. The fresh weight of the suspension of embryonic tissue increased in the 4% glucose treatment at a maximum of 11.3 during a 12 day period of culture. In both with or without BAP treatments there was a strong positive correlation between dry weight and sucrose concentration ($r = 0.88$ and $r = 0.86$, Fig. 13, respectively). However, there was a negative correlation between fresh weight and glucose concentration ($r = -0.81$ and $r = -0.99$, Fig. 14, respectively).

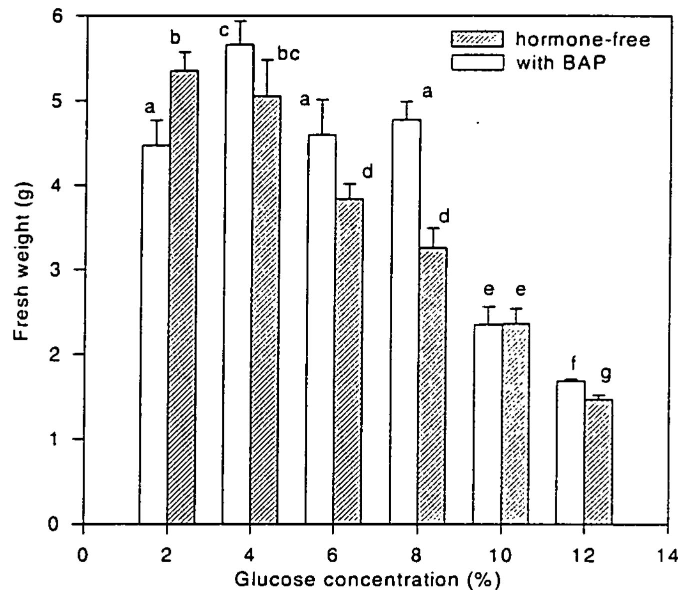


Fig. 14 : Relationship between glucose concentration (%) and fresh weight of EMS (g) on media supplemented with 1 mgl^{-1} BAP and on hormone-free media. Different lowercase letters above any two columns indicate that these two means are statistically different at $p = 0.05$ according to the Student t-test. The data for the mean of two experiments with 5 replicates. Embryonic tissues had been subcultured 5 times before the experiments performed. Vertical bars represent the standard error of the mean.

4.1.4.3 : Effects of fructose on dry matter content and fresh weight in EMS cultures of pistachio

In this experiment, the effect of fructose, another important media component, was studied. The effect of fructose was tested in Erlenmeyer flasks containing 50 ml of MS medium supplemented with or without 1 mgl^{-1} BAP, and with the following concentrations (w/v) of fructose: 2%, 4%, 6%, 8%, 10% and 12%.

The effects of fructose concentrations on dry matter are shown in Fig 15. Statistical analysis of data showed that there were highly significant differences in the frequencies of dry matter

content of EMSes between the tested treatments (P < 0.001). There was a steady increase in the frequencies of dry matter content of the embryonic mass on both media with or without BAP, and with an increase in the concentrations of sucrose up to a concentration of 10% of fructose and a slight decrease thereafter (Fig. 15).

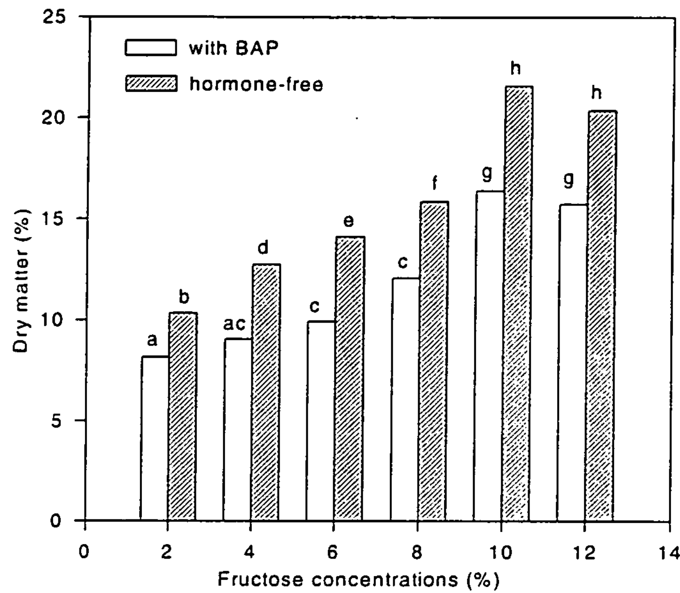


Fig. 15 : Relationship between fructose concentration (%) and dry matter of EMS (%) on media supplemented with 1 mgl^{-1} BAP and on hormone-free media. Different lowercase letters above any two columns indicate that these two means statistically different at p = 0.05 according to the Student t-test. The data for the mean of two experiments with 5 replicates. Embryonic tissues had been subcultured 6 times before the experiments performed.

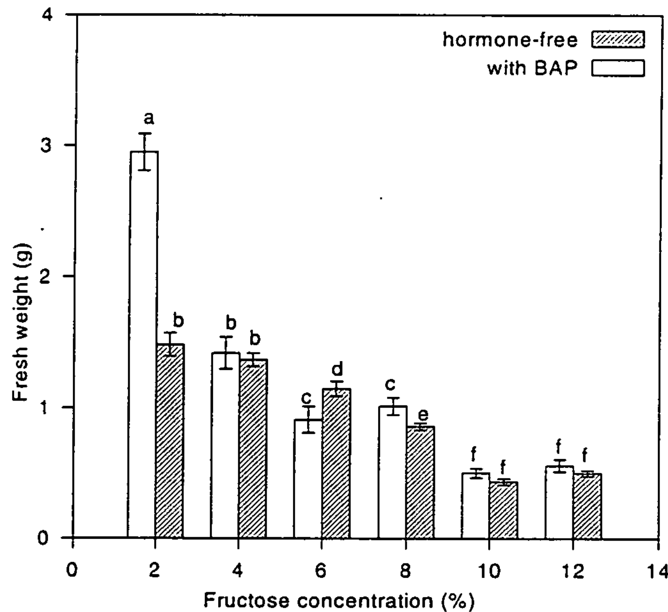


Fig. 16 : Relationship between fructose concentration (%) and fresh weight EMS (g) on media supplemented with 1 mgl^{-1} BAP and on hormone-free media. Different lowercase letters above any two columns indicate that these two means are statistically different at p = 0.05 according to the Student t-test. The data for the mean of two experiments with 5 replicates. Embryonic tissues had been subcultured 6 times before the experiments performed. Vertical bars represent the standard error of the mean.

Cultures grown on a growth regulator-free media attained approximately 30% higher dry matter than cultures grown on BAP treated media. With increasing fructose the fresh matter production declined but dry matter production increased. However, non-embryonic and black tissue clusters were also observed in all treatments after 10 days of culture. Apparently there was decreasing cell division because there was visible black mucilaginous material secreted by the embryonic tissues that was very evident in all treatments. The ratio of final to initial fresh weight of the fructose embryonic tissue ranged from 0.86 to 5.88 on MS medium supplemented 10% and 12% fructose, respectively (Fig.16). In both with or without BAP treatments (r = 0.95 and r = 0.95, respectively) there was a strong positive correlation (Fig.15) between dry weight and fructose concentration. However, there was a negative correlation (Fig. 16) between fresh weight and fructose concentration (r = - 85 and r = - 0.97, respectively). Therefore, fructose was not tested in further studies.

4.1.4.4 : Effects of lactose on dry matter content and fresh weight in EMS cultures of pistachio

In this experiment, the effect of lactose, another important media component, was studied. The effect of lactose was tested in Erlenmeyer flasks containing 50 ml of MS medium supplemented with or without 1 mgl^{-1} BAP, and with the following concentrations (w/v) of lactose: 2%, 4%, 6%, 8%, 10% and 12%.

The effects of lactose concentrations on dry matter are shown in Fig. 17. Analysis of data indicated that there was significant difference on the dry matter content as well as on fresh weight of EMSes between the treatments. The dry matter content increases with an increasing concentration of lactose with or without BAP (Fig. 17). On the contrary, the fresh weight of EMSes increased up to 6% of lactose and thereafter decreased gradually (Fig. 18). The ratio of final to initial fresh weight of the lactose treated embryonic tissues ranged from 1.3 to 2,86 fold on MS medium supplemented with 12% and 4% lactose, respectively.

As in the fructose case, all lactose treatments tested produced visible black material which inhibited the embryonic masses very evident in all treatments. In both with or without BAP treatments, there was a strong positive correlation between dry weight and lactose concentration r = 0.93 and r = 0.87, respectively. However, there was a negative correlation (Fig. 18) between fresh weight and lactose concentration with growth regulator-free medium (r = - 0.90) and values in BAP supplemented treatments were unrelated (r = - 0.14). Overall, from the above results it was concluded that the medium supplemented with sucrose generally gave the best performance. More to the point, the viability of embryogenic masses could only be maintained when transferred from sucrose-to sucrose and sucrose to glucose supplemented media. The

generation of phenols, however, was also observed in glucose treatments. Among the carbohydrate variants tested in the first step of cultures, differentiation was partially inhibited by lactose and fructose and it was completely inhibited by mannitol, sorbitol, polyethleneglycol (PEG) ribose and xylose with or without 1 mgl^{-1} BAP.

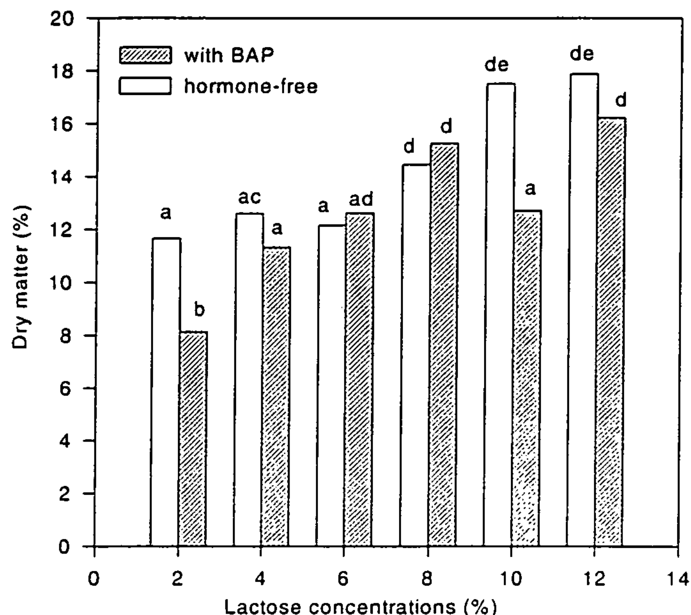


***Fig. 17**: Relationship between lactose concentration (%) and dry weight of EMS (%) on media supplemented with 1 mgl^{-1} BAP and on growth regulator-free media. Different lowercase letters above any two columns indicate that these two means are statistically different at p = 0.05 according to the Student t-test. The data for the mean of two experiments with 5 replicates. Embryonic tissues had been subcultured 6 times before the experiments performed.*

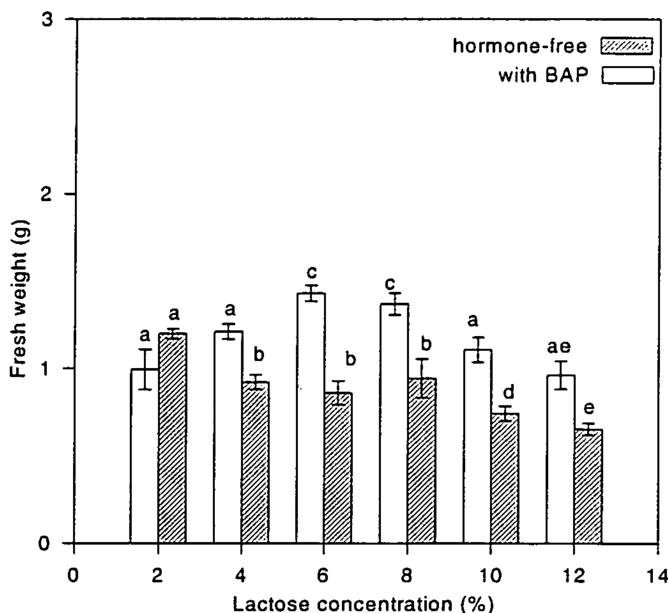


***Fig. 18** : Relationship between lactose concentration (%) and fresh weight of EMS (g) on media supplemented with 1 mgl^{-1} BAP and on hormone-free media. Different lowercase letters above any two columns indicate that these two means are statistically different at p = 0.05 according to the Student t-test. The data for the mean of two experiments with 5 replicates. Embryonic tissues had been subcultured 6 times before the experiments performed. Vertical bars represent the standard error of the mean.*

On the basis of these results it could be postulated that sucrose possibly had a conditioning effect at the initial step of the culture. Thus, sucrose could be used as a carbohydrate source for the proliferation of pistachio embryonic mass cultures. Further studies will also be conducted to find out the optimal concentration of sucrose for the maturation of immature somatic embryos in the liquid and solidified cultures in the following subsections.

4.1.4.5 : Effects of basal salts formulation on dry matter content and fresh weight in EMS cultures of pistachio

The aim of this experiment was to determine the optimum salts formulation for growth, fresh and dry weight in suspension cultures of pistachio. Four basal salts formulations were used, i.e. MS, WP, SH and G-5. Ten-day-old clusters of cells previously grown in MS medium were suspended in the four different media. A 50 ml aliquot of each suspension containing 0.5 g FW of clusters of cells was inoculated into each 250 ml Erlenmeyer flask containing the respective medium. All media were supplemented with 500 mgl^{-1} casein hydrolysate, and 50 mgl^{-1} l-ascorbic acid, and their pH adjusted to 5.7. Each treatment consisted of 5 replicates. Cultures were placed on a shaker rotating at 98 rpm at 25°C and under 20 $\mu mol.\ m^{-2}\ s^{-1}$ of light. After 10 d, the cultures were harvested and their fresh and dry weight contents determined according to the methods described in the previous sub-section.

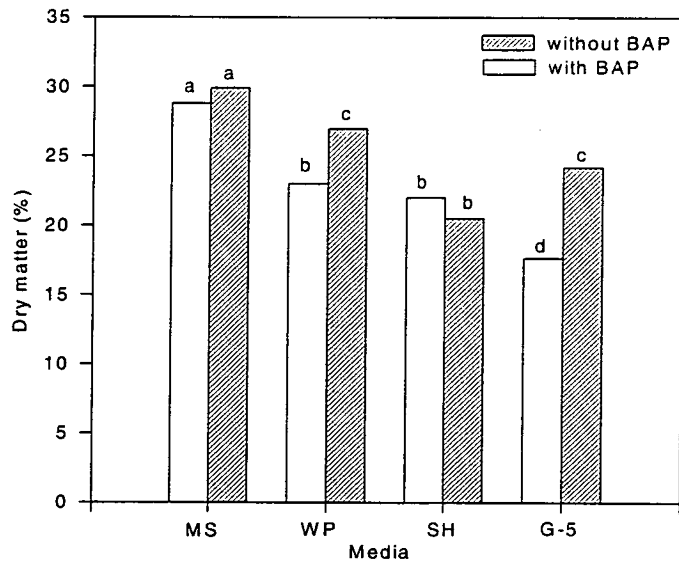


Fig. 19 : Effects of different basal salts formulation (MS, WP, SH and G-5 media) on dry matter content of embryogenic suspension cultures of pistachio. Different lowercase letters above any two columns indicate that the means are statistically different at p = 0.05 according to the Student t-test. Data are means of two experiments with 5 replicates. Embryogenic mass had previously been subcultured 7 times before the experiments were performed.

Statistical analysis of the results showed that the dry matter content and fresh weight were significantly influenced by the composition of the basic media (Figs. 19 and 20). From the results presented in Fig. 19, it would appear that MS is superior to the others in terms of its ability to support dry matter content, and is followed closely by WP and G-5 media. SH medium supports dry matter to the same extent but less than WP and G-5 media. In terms of the fresh weight of EMS, MS medium was again superior to the others (Fig. 20). Visual observation of the cultures showed that those in MS medium were the most friable proliferating, followed by WP, SH and G-5 media respectively. MS medium was then chosen as the standard medium for future experimentation because it was found to support proliferation as well as dry matter content better than the others.

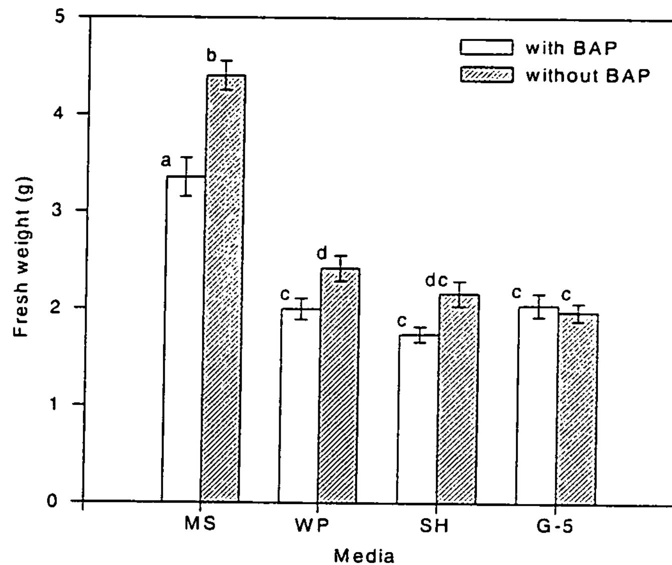


Fig. 20 : Effects of different basal salts formulation (MS, WP, SH and G-5 media) on the growth of embryogenic suspension cultures of pistachio. Different lowercase letters above any two columns indicate that the means are statistically different at p = 0.05 according to the Student t-test. Data are means of two experiments with 5 replicates. Embryogenic mass had previously been subcultured 7 times before the experiments were performed. Vertical bars represent the standard error of the mean..

4.1.4.6 : The effects of light intensity on dry matter and fresh weight of EMS

The aim of this experiment was to ascertain the extent to which EMS proliferation is influenced by light intensity. Cultures were set up as described in sub-section 4.1.3. using the previously determined optimum conditions. A range of light intensities (10, 20 and 25 μmol. m^{-2} s^{-1}) were achieved simply by placing cultures at varying distances from the light source. Light intensity was measured using a Photometer. The three light intensities were evaluated for dry matter and fresh weight on the cultures incubated in the presence or absence of BAP (2 mgl^{-1}). Fresh measurements were done at d 10, and the dry matter content was determined by the methods described in sub-section 4.1.3.

Table 36 : The effects of three levels of light intensity on the dry matter content of EMS cultures of pistachio*.

Light intensity (μmol. $m^{-2} s^{-1}$)	Dry matter content (%)	
	Cultured with BAP	Cultured without BAP
10	17.9 ± 0.7 a	22.4 ± 1.0 a
20	23.0 ± 0.7 b	27.1 ± 0.7 b
25	21.5 ± 0.8 b	28.5 ± 1.1 b

**The mean number of SEs is shown ± the sample standard errors of the mean values from two different experiments. Mean separation using the Student t-test, means with different lowercase letters are significantly different at p = 0.05.*

Table 37 : The effects of various levels of light intensity on the fresh weight of EMS cultures of pistachio*.

Light intensity (μmol. $m^{-2} s^{-1}$)	Fresh weight (g)	
	Cultured with BAP	Cultured without BAP
10	1.7 ± 0.0 a	1.9 ± 0.0 a
20	2.0 ± 0.1 b	3.3 ± 0.2 b
25	2.2 ± 0.1 b	4.5 ± 0.1 c

**The mean number of SEs is shown ± the sample standard errors of the mean values from two different experiments. Mean separation using the Student t-test, means with different lowercase letters are significantly different at p = 0.05.*

The results presented in Table 36 show that light intensity had a significant influence of on the dry matter content of EMS cultures of pistachio. The cultures subjected to an intensity of 25 μmol. $m^{-2} s^{-1}$ gave the highest dry matter content, while cultures exposed to 10 μmol. $m^{-2} s^{-1}$ of light intensity gave the least. The cultures subjected to 10 μmol. $m^{-2} s^{-1}$ of light intensity produced significantly lower dry matter content on both media with or without BAP. There was no significant difference in the dry matter content of cultures between the 20 and 25 μmol. $m^{-2} s^{-1}$ treatments. The results presented in Table 37 show that light intensity significantly influenced the fresh weight of EMS cultures of pistachio. The cultures subjected to 25 μmol. $m^{-2} s^{-1}$ of light intensity gave the highest fresh weight, while cultures exposed to 10 μmol. $m^{-2} s^{-1}$ gave the least. There was no difference in fresh weight between 20 an 25 μmol. $m^{-2} s^{-1}$ treatments.

4.1.5 : Maturation of SEs

Maturation experiments were carried out both in liquid and on agar-solidified media. Unless otherwise stated maturation studies were carried out on an agar-solidified medium. The maturation experiments were carried out with only one embryogenic line. The embryogenic potential of the EMS is defined as the number of SEs produced per 250 mg fresh weight of the

EMS. The growth regulators (BAP and ABA) were filter sterilised and, if used were added to cooled media after autoclaving. After 4 weeks of culture unless otherwise stated, the SEs were counted by eye. Unless otherwise stated, in the studies in the following sub-sections, all media were also supplemented with 500 mgl^{-1} casein hydrolysate, 50 mgl^{-1} l-ascorbic acid, 2 mgl^{-1} BAP, 4% sucrose and 0.7% agar, and the pH medium was adjusted to 5.7 before autoclaving. The cultures were placed under cool-white fluorescent light (25 µmol m^{-2} s^{-1}, continuous photoperiod) at 25°C. A randomised complete block design was used for the maturation experiments. A General Linear Model (GLM) was performed and the Student *t*-test adjusted for the different number of observations used in a pairwise comparison of treatments. Each treatment used two blocks, 5 replicates (Petri dish) per block and 5 explants (ca. 50 mg) per replicate.

4.1.5.1 : Effects of media type on maturation of SEs

Ten days after subculture on the proliferation medium, pieces of actively growing EMS were transferred onto agar-solidified MS, WP, SH and G-5 media (all media tested were obtained from Sigma Ltd. and the contents of all media was given at Table 20). Each medium was made up at the manufacturer's recommended strength. All cultures were incubated under conditions described in section 4.1.5.

After 4 weeks of culture, the effects of the media became evident in the number of SEs developed (Table 38). SEs were produced on all tested media (MS, SH, WP and G-5). However, the G-5 medium appeared to have an inhibiting effect on embryo maturation, since the mean number of embryos per 250 mg embryogenic mass was significantly lower on G-5 medium. Many explants on all media produced swollen embryos or recallusing tissues and failed to mature embryos. The MS medium gave the highest number of mature SEs after four weeks of culture, but there was no significant difference between the responses on MS and SH media.

Regardless of medium, all pistachio SEs appeared to arise from proliferated embryogenic masses. From the present study it would appear that the MS medium is superior to the others in terms of the number of mature SEs after 4 weeks of culture and is closely followed by SH and WP media, which support maturation to the same extent. When the cultures were maintained on the same medium for another four weeks, the number of embryos increased. However, early matured embryos started to swell and failed to extend their hypocotyls. From these results, it is clear that the MS medium increased for maturation of SEs. Therefore, the MS medium was now chosen for further studies.

Table 38 : Number of SEs per 250 mg fresh weight of EMS of pistachio obtained on different media after 4 weeks of culture.

Media	Mean number of somatic embryos ± SE
Murashige and Skoog (MS)	14.25 ± 0.71 a
Schenk and Hildebrandt (SH)	12.15 ± 0.91 ab
Woody plant medium (WP)	11.75 ± 0.71 b
Gamborgh's B-5 (G-5)	8.85 ± 1.08 c

Only visible SEs with cotyledons were counted by eye. The mean number of SEs is shown ± the sample standard errors of the mean values from two different experiments. Means were separated using the Student t-test. Means with different lowercase letters are significantly different at p = 0.05.

4.1.5.2 : Effects of strength of MS mineral salts medium on number of SEs

Ten days after subculture on the proliferation medium, pieces of actively-growing EMS were transferred onto different strengths of the MS medium (2/1, 1/1, 1/2 and 1/4). All cultures were incubated under conditions described in section 4.1.5.

Table 39 : Number of SEs per 250 mg fresh weight of EMS of pistachio obtained on different strengths of MS medium after 4 weeks of culture.

MS medium strength	Mean number of somatic embryos ± SE
1/4	11.30 ± 0.78 a
1/2	12.80 ± 0.90 ab
1/1	15.05 ± 1.07 b
2/1	5.22 ± 0.66 c

Only visible SEs with cotyledons were counted by eye. The number of SEs is shown ± the sample standard error from two different experiments. Mean separation using the Student t-test. Means with different lowercase letters are significantly different at p = 0.05 (5% level).

After 4 weeks of culture, the numbers of matured SEs produced showed differences on the different strength of the agar-solidified MS medium (Table 39). The number of SEs per 250 mg embryogenic mass was significantly greater on full and half strength MS medium than on double strength MS medium. Many explants on the double strength MS medium were inhibited. The highest number of SEs was obtained on the full strength MS medium. There was no significant difference between the responses on half and full strength MS medium. Based on these results, the full strength MS medium was then chosen for the maturation investigations in the subsequent sections.

4.1.5.3 : Effect of carbohydrates on the maturation of SEs

Ten days after subculture on the proliferation medium, pieces of actively-growing EMS were transferred onto full strength MS medium supplemented with sucrose, glucose, fructose, mannitol and PEG each at 4%, with or without 2 mgl^{-1} BAP. All cultures were incubated under conditions described in section 4.1.5.

After four weeks of culture, the effects of these carbohydrates upon embryo maturation are shown in Table 40. Analysis of variance indicated very highly significant ($P < 0.001$) differences in the effects of carbohydrates on SE maturation. Sucrose, in the presence of BAP in terms of the number of SEs obtained, was superior to other tested carbohydrates but not as efficient as glucose in the absence of BAP. By contrast embryogenic masses cultured in the presence of mannitol and PEG browned within 3 days of the start of incubation, and SEs were not produced in the presence of mannitol and PEG in the culture media. These results suggest that the type of carbohydrate available as a carbon source had a significant effect on the maturation of SEs. Addition of BAP to the culture medium affected the number of matured SEs after 4 weeks of culture.

Table 40 : Number of SEs per 250 mg fresh weight of EMS of pistachio obtained on different carbohydrate sources after 4 weeks of culture.

Carbohydrate type (4%, w/v)	Mean number of SEs ± SE	
	With 2 mgl^{-1} BAP	Without BAP
Sucrose	23.2 ± 1.7 a	15.9 ± 0.9 a
Glucose	14.2 ± 1.5 b	19.6 ± 1.4 b
Fructose	9.9 ± 1.4 c	6.6 ± 1.4 c

SEs with cotyledons were counted by eye. The number of SEs is shown ± the sample standard errors from two different experiments. Means were separated using the Student t-test. Means with different lowercase letters are significantly different at p = 0.05.

Overall, most SEs were obtained with sucrose as a carbon source in the presence of 2 mgl^{-1} BAP. In the presence of sucrose, embryos produced with the most normal morphology resembling zygotic embryos. Mannitol and PEG which are not metabolised by EMS to any great extent, did not lead to somatic embryogenesis. These results suggest that sucrose together with BAP is the optimum choice of carbon source for the development of SEs. In the following experiments the effect of sucrose and BAP concentration will be studied in more detail.

4.1.5.4 : Effects of cytokinin and auxin type on pistachio SE maturation

The objective of this experiment was to evaluate the effects of the cytokinins BAP, BA, K, TDZ, 2iP and Zea and the auxins 2,4-D, NAA, IAA, IBA, Picloram and Dicamba, each at 2 mgl^{-1}, on the

maturation of pistachio SEs from actively growing EMS. The embryogenic potential of EMS in the treatments applied here is defined as the number of SEs per 250 mg fresh weight of the EMS after 4 weeks of culture. All cultures were incubated under conditions described in section 4.1.5.

Cytokinin was a very highly significant source of variance for SE maturation as demonstrated by analysis of variance of the data from this experiment (Table 41, $P < 0.001$). All explants including those on the growth regulator-free medium produced SEs. However, BAP was superior to all other PGRs tested inducing a mean of 34.95 SEs at 2 mgl^{-1}. Indeed, regardless of the maturation medium, all pistachio SEs appeared to arise directly from proliferated EMS. On the growth regulator-free medium (control) the mean number of the somatic embryos was not significantly lower to that observed with BAP medium, but higher than observed with any other PGR.

Table 41: Number of SEs per 250 mg fresh weight of EMS of pistachio obtained on different cytokinins* or auxins after 4 weeks of culture on MS medium.

Cytokinins (2 mgl^{-1})	Mean number of SEs ± SE	Auxins (2 mgl^{-1})	Mean number of SEs ± SE
Control	30.5 ± 1.1 a	Control	25.9 ± 0.9 a
BAP	34.9 ± 0.3 a	2,4-D	13.1 ± 0.7 b
BA	10.0 ± 0.8 b	NAA	8.6 ± 0.8 c
Kin	15.8 ± 0.1 c	IAA	8.4 ± 0.6 c
TDZ	6.6 ± 0.6 d	IBA	12.7 ± 0.7 b
2iP	12.0 ± 0.6 b	Picloram	14.3 ± 0.9 b
Zea	6.8 ± 0.5 d	Dicamba	13.0 ± 0.7 b

**Cytokinin and auxin experiments are conducted on different days. SEs with cotyledons were counted by eye. The number of SEs is shown ± the sample standard errors from two different experiments. Means were separated using the Student t-test. Means with different letters are significantly different at $p = 0.05$.*

TDZ and Zea appeared to have strongly and significantly inhibiting effect on embryo maturation. Most of the explants on TDZ and Zea maturation medium proliferated EMS over time and produced few swollen embryos, and generally failed to develop embryos. The mean yield of SEs was significantly greater in BA, 2iP and Kin media than in media with TDZ and Zea. The number of SEs generally increased with time when the maturation period was extended due to the proliferation of EMS. SE developed on BAP treatments were morphologically similar. From these results it may be concluded that the use of the cytokinins Kin, 2iP, Zea and TDZ on the maturation medium reduced the embryo numbers or embryo morphology compared the PGR-free medium. Based on the above results the cytokinin BAP was then chosen to study the effects of various BAP concentrations during maturation on pistachio somatic embryogenesis.

There were highly significant differences ($P < 0.001$) between the mean numbers of matured SEs per 250 mg EMS for the different auxins (Table 41). A mean of 25.9 SEs was obtained with the growth regulator-free medium and no auxins induced greater numbers. A mean of 14.3 somatic embryos was obtained on Picloram supplemented medium. Picloram induced more SEs than IAA or NAA but was not significantly more than 2,4-D, IBA or Dicamba IAA and NAA were least effective auxins at inducing SEs. In these studies, high mean numbers of SEs with a healthy appearance were of high priority. Embryo quality and form did not benefit from the use of different auxin types in this system. Therefore no further studies with auxins on maturation of SEs were carried out.

4.1.5.5 : Effects of ABA in agar-solidified MS medium on the maturation of SEs

The aim of this experiment was to determine the influence of ABA and sucrose on the development of mature SEs. Ten days after subculture on the proliferation medium, pieces of actively growing EMS were transferred onto agar-solidified MS medium containing the following combinations of ABA and sucrose:

0.25 mgl^{-1} ABA + 2 %, 4%, 6% or 8 % sucrose

0.5 mgl^{-1} ABA + 2 %, 4 %, 6% or 8 % sucrose

1.0 mgl^{-1} ABA + 2 %, 4 %, 6% or 8 % sucrose

2.0 mgl^{-1} ABA + 2 %, 4 %, 6% or 8 % sucrose.

All cultures were incubated under conditions described in section 4.1.5.

4.1.5.5.1 : Quantitative aspects of SE development in ABA/sucrose treatments

Analysis of variance showed significant effects of sucrose and ABA concentrations on the number of mature SEs, and more importantly, revealed an interaction between ABA concentration and sucrose concentration (Table 42).

The highest quantities of SE were observed on media with 0.5 to 1 mgl^{-1} ABA and 4%, 8% sucrose (24.00 and 27.23 per 250 mg fresh weight respectively); when high concentrations of ABA (2 mgl^{-1}) were combined with 2%, 4% and 6% sucrose, the number of SEs was reduced. In addition, this reduction was also seen when a low concentration of ABA (0.25 mgl^{-1}) was combined with different concentrations of sucrose because there were only minor differences in the number of SEs produced at 2%, 4%, 6% and 8% sucrose combined with 0.25 mgl^{-1} ABA.

In general the mid concentrations of ABA (0.5 and 1 mgl^{-1}) and the higher sucrose concentrations were most favourable for a high frequency of matured SEs, whereas at 0.5 mgl^{-1} ABA an increase in sucrose concentration from 2% to 8% reduced the number of SEs.

Table 42 : Number of mature SEs per 250 mg fresh weight of pistachio embryogenic mass obtained on solid media with different ABA and sucrose concentrations after 4 weeks of culture.

	Sucrose(%)			
ABA mgl^{-1}	2%	4%	6%	8%
0.25	11.40 a	11.80 a	11.60 a	15.10 ab
0.50	20.00 b	24.00 bc	19.60 b	10.40 a
1.00	11.60 a	16.10 ab	14.40 a	27.23 c
2.00	9.86 a	15.60 a	14.20 a	16.80 ab

Mature SEs with cotyledons were counted by eye. Means followed by the same letter are not significantly different at p = 0.05 based on the Student t-test.

From these results, it may be concluded that ABA at 0.5 to 1.0 mgl^{-1}, with 4% to 8% sucrose may be chosen for the maturation of SEs in agar-solidified MS medium.

4.1.5.5.2 : Morphological aspects of SE development in ABA/sucrose treatments

The proliferation ability of EMS was affected by the sucrose concentration and by the ABA concentration. The EMS continued to proliferate on media with all the concentrations tested but showed reduction of growth or partial inhibition on the medium with 2 mgl^{-1} ABA. After 3 weeks of culture, the SEs underwent stages of late embryogeny and by 4 weeks of culture, the effects of ABA and sucrose became evident not only in the number of SEs developed, but most importantly in the morphological features. On media supplemented 2 mgl^{-1} ABA, the decrease of the sucrose concentration from 8% to 2% resulted in an inhibition of SE development. However, in the presence of 2 mgl^{-1} ABA with higher concentrations of sucrose (6% and 8%), SEs also matured well and they had elongated hypocotyl and cotyledons (Onay et al. 1995). In contrast, SEs obtained on media with BAP, some of the treatments had started radicle growth during the 4 weeks of culture. In other words, precocious germination was not noted but the number of the matured SEs were lower than in the BAP treatments.

4.1.5.6 : Effects of ABA, BAP and sucrose in liquid medium on the maturation of SEs

The aim of these experiments was to investigate the positive effect of ABA, BAP and sucrose on the development of mature SEs in the liquid MS medium. Ten days after subculture on the proliferation medium, pieces of actively-growing EMS were transferred into liquid MS medium with combinations of ABA and BAP at 0, 0.5, 1.0 and 2.0 mgl^{-1} together with sucrose at 2, 4, 6, or 8% w/v, and the cultures were incubated under continuous light (25 $\mu mol\ m^{-2}\ s^{-1}$) at 25°C. ABA was filter-sterilised and added to the media after autoclaving. Cultures of EMS were placed in liquid MS medium using 250 ml Erlenmeyer flasks continuously rotated at 98 rpm in

continuous light at 25°C. Each experiment was performed twice. After 2 or 3 weeks of culture, the SEs were counted by eye.

4.1.5.6.1 : Effects of BAP on the maturation of SEs

Pieces of EMS transferred to liquid maturation media exhibited changes in the proliferation ability and progressive development of SEs. The colour of the clusters of EMSes was generally greyish especially in the control treatment, but was deep green in the BAP supplemented treatments. The proliferative ability was affected by the sucrose concentration and BAP concentration. The EMSes continued to proliferate in all treatments tested but showed reduced growth on the medium with 8% sucrose and partial inhibition of growth on the medium with 2 mgl^{-1} BAP. Seven days after the culture, the effects of BAP and sucrose were not clear, but the SEs underwent stages of late embryogeny in all tested treatments. After 2 and 3 weeks of culture, SEs had developed cotyledons. At this time, the effects of BAP and sucrose were evident in the number of SEs developed. On media without BAP, generally the SEs were greyish or yellowish, had elongated hypocotyls and cotyledons. In the BAP treatments, in some flasks, precocious germination was noted (if an embryo started radicle growth, it was accepted as a germinating or a germinated embryo). However, none of the control treatments produced any precociously germinated SE during 2 or 3 weeks of culture in liquid medium.

Analysis of variance showed significant effects of sucrose and BAP concentrations on the number of mature SEs. Interaction was also evident between BAP concentration and sucrose concentration. Table 43 shows the mean numbers of mature SEs on the liquid MS media supplemented with different BAP and sucrose concentrations. The number of SEs on 0.5 mgl^{-1} BAP and the control treatments with sucrose concentrations 2% and 6% was significantly higher than on the remaining treatments (Table 43), respectively. Media with 0.5 mgl^{-1} BAP and 2% sucrose induced the most embryos (50.1 per 250 mg fresh weight). Increasing the BAP concentration from 0.5 to 2.0 mgl^{-1} resulted in generally less mature SE. Sucrose concentrations above 6% were not beneficial. The highest concentrations of BAP and sucrose produced the lowest yields of SEs. From these results presented in Table 43, it may be suggested that the maturation of SEs could be achieved on a lower concentration of BAP i.e. 0.5 mgl^{-1} BAP or growth regulator-free-liquid MS medium.

However, at this stage the quality of SEs cannot be predicted because these SEs may remain arrested in the present culture system. Therefore, in the following experiment, a study will be carried out using similar concentrations of ABA and sucrose in order to make a comparison between BAP and ABA.

Table 43 : Number of mature SEs per 250 mg fresh weight of pistachio embryogenic mass obtained on media with different BAP and sucrose concentrations after 2 weeks in liquid medium.

	Sucrose(%, w/v)			
BAP mgl^{-1}	2%	4%	6%	8%
0.0	35.6 ± 2.29 c	39.2 ± 2.47 bc	49.8 ± 3.20 a	33.3 ± 2.23 cd
0.5	50.1 ± 3.40 a	48.4 ± 2.98 a	42.8 ± 1.97 b	40.5 ± 2.30 b
1.0	34.5 ± 1.91 c	40.1 ± 2.28 b	36.7 ± 1.88 c	41.1 ± 2.76 b
2.0	38.5 ± 2.36 c	35.9 ± 2.35 c	32.8 ± 1.97 d	29.7 ± 2.12 d

Mature SEs with cotyledons were counted by eye. Means followed by the same letter are not significantly different at p = 0.05 based on the Student t-test.

4.1.5.6.2 : Effects of ABA in liquid medium on the maturation of SEs

As in the cytokinin BAP treatments, pieces of EMS transferred to liquid maturation media exhibited change in the proliferation ability and progressive development of SEs in the ABA treatments. The proliferative ability was affected both by sucrose concentration and ABA concentration (Table 44). By day 7, pieces of actively growing EM transferred to the maturation medium had increased in size enormously. The colour of the clusters of EMSes was generally greyish or slightly yellowish especially in the control treatment, and deep green in the ABA supplemented treatments. The EMSes showed sustained proliferation in all treatments tested but partial inhibition of growth on the medium with 1 or 2 mgl^{-1} ABA. After 7 days of culture, the effects of ABA and sucrose were clear and the SEs underwent stages of late embryogeny in the control and 0.5 mgl^{-1} ABA treatments tested. After 2 weeks of culture, the developing embryos started developing elongated cotyledons. At this time the effects of ABA and sucrose became evident in the number of SEs developed. None of the treatments tested produced any precociously germinated SEs during 2 or 3 weeks of culture in the liquid medium.

Analysis of variance showed significant effects of sucrose and ABA concentrations on the number of mature SEs. An interaction was also evident between ABA concentration and sucrose concentration. Table 44 shows the mean number of mature SEs on the liquid MS media supplemented with different ABA and sucrose concentrations. There was significant difference in the mean number of mature SEs between the treatments tested (Table 44). The number of SEs on 0.5 mgl^{-1} ABA with sucrose concentrations 4% was significantly higher ($P < 0.05$) than on the remaining treatments (Table 44). Media with 0.5 mgl^{-1} ABA and 4% sucrose induced the most embryos (50.7 per 250 mg fresh weight). Increasing the ABA concentration from 0.5 to 2.0 mgl^{-1} resulted in less mature SEs. ABA concentration above 0.5 mgl^{-1} were not beneficial. Sucrose concentrations 2% and 4% were the most beneficial treatments. Further increases in the

sucrose concentration above 4% had an inhibiting effect, so that the highest concentrations of ABA and sucrose produced the lowest yields of SEs.

Table 44 : Numbers of mature SEs per 250 fresh weight of pistachio embryogenic mass obtained on media with different ABA and sucrose concentrations after 2 weeks in liquid medium.

	Sucrose(%)			
ABA mgl^{-1}	2%	4%	6%	8%
0.0	35.8 ± 2.12 c	35.8 ± 2.24 c	46.3 ± 3.69 ab	34.5 ± 1.99 c
0.5	46.5 ± 2.90 ab	50.7 ± 2.72 a	41.7 ± 2.10 b	34.6 ± 2.08 c
1.0	22.6 ± 1.96 d	18.1 ± 1.41 e	19.4 ± 1.50 de	16.7 ± 1.05 e
2.0	16.7 ± 1.73 e	23.6 ± 1.00 d	17.6 ± 2.13 e	23.5 ± 4.19 d

Mature SEs with cotyledons were counted by eye. Means followed by the same letter are not significantly different at p = 0.05 based on the Student t-test.

From results presented in Table 44, it may be concluded that the maturation of SEs was best achieved on low concentrations of ABA i.e. 0.5 mgl^{-1} ABA or PGR-free-liquid MS medium. Moreover, there was partial inhibition of cultures in tested treatments (i.e. 1 and 2 mgl^{-1} ABA). In the following subsections, a study will be carried out in order to determine the effects of the maturation treatments on the quantity of SE germination and subsequent plantlet development from SEs matured using growth regulator-free MS medium.

4.1.6 : *IN VITRO* GERMINATION AND DEVELOPMENT OF THE EXCISED MATURE ZYGOTIC EMBRYOS OF PISTACHIO, *P. VERA* L.

Up to now, little is known of the effect of culture factors on germination of zygotic embryos of pistachio *in vitro*. The aims of these experiments were to establish the germination and development characteristics of zygotic embryos of *P. vera* in culture, using the sterile-excised embryos as a primary explant system; to apply the knowledge obtained to the development of optimal culture conditions for SE germination *in vitro*; then to compare growth patterns and requirements of somatic and zygotic embryos. The effects of the media (MS, WP, G-5 and SH), cytokinins (BAP and Kin), auxins (IBA and NAA), ABA, sugars (sucrose, glucose and fructose) and the effect of the light and dark regime were studied in a series of 4 experiments. Unless stated otherwise, data are based on 15 isolated zygotic embryos per treatment. If not otherwise noted, experiments were done with MS medium supplemented with 4% sucrose, 500 mgl^{-1} casein, 50 mgl^{-1} l-ascorbic acid and 0.7% agar.

The following criteria were used to screen for best growth: the percentage of responding embryos, embryo length, embryo colour, surface structure of the shoots, and length of the root.

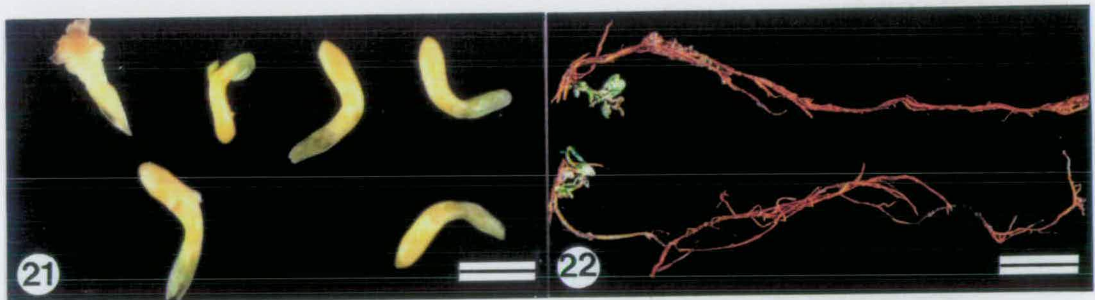
Embryo germination and growth were evaluated after a 2 week incubation period. The zygotic embryos used in these experiments were fully developed, having reached maturity about 6 months after harvesting. To reduce variability due to small and ill-formed embryos, which are least likely to grow normally, equal sized embryos were chosen as far as possible. In a normal healthy excised embryo; the tigellum is pale green and the haustorium is yellow in colour. Embryos were incubated for 14 days.

4.1.6.1 : Seed and embryo morphology

Seed size in the study species varied from 12 to 15 mm. Extraction of embryos was relatively simple after kernels had been surface sterilised. Embryos are normally located at the attachment end of the kernels which contain endosperm. Embryos are approximately 7-9 mm. Figure 21 shows a group of excised zygotic embryos. The embryos have well developed apical and root meristems, two fully differentiated leaves and the rudiments of a third. Embryos, at excision, have a creamy yellow coloured tigellum and a polar almost white haustorium.

4.1.6.2 : Changes in morphology associated with the *in vitro* development of excised embryos

Zygotic embryo germination in pistachio may be separated into two phases: root elongation that occurs within 24 h after incubation and a shoot elongation phase that starts three days after initiation and continues over the culture period.



Figs. 21-22 : *Germination of zygotic embryos:* ***Fig. 21 :*** *shows a group of zygotic embryos, bar = 4 mm.* ***Fig. 22 :*** *Germinated zygotic embryos two weeks after incubation, bar = 45 mm.*

In general, radicle extension had occurred within 24 h. By day 3 a leaf sheath had been formed and by day 5 the first leaf had emerged through the leaf sheath. Leaf development was slower than that of the root, but within 10 days a second or third leaf has emerged and associated

adventitious roots were clearly discernible (Fig. 22). By day 14 the excised embryo has at least two distinct green leaves, an elongated radicle and some adventitious roots.

4.1.6.3 : The effect of media on germination of zygotic embryos of *P. vera*

The media tested were MS, WP, G-5, and SH separately each at suggested concentration (by manufacturer, mgl^{-1}). Embryo germination was influenced by the type of medium (Table 45). There was no evidence of significant difference in the frequencies of germinated embryos between media tested.

Table 45 : Effect of media on germination of zygotic embryos of *P. vera*.

Treatments	% of germinated embryos	Mean shoot length (mm) ± SE	Mean root length (cm) ± SE
MS	87	10.63 ± 1.07 a	34.09 ± 5.49 a
WPM	80	11.46 ± 1.21 a	36.69 ± 7.03 a
SH	74	7.90 ± 1.10 b	23.72 ± 3.97 b
G-5	74	10.50 ± 0.96 a	40.41 ± 5.66 a

Data recorded on the 14 th day of incubation represents an average of 15 replicates per treatment. Means in a column followed by the same letters are not significantly different at p = 0.05 according to the Student t-test.

The proportion of germination was highest (87%) in embryos cultured on MS medium and gradually decreased to 80%, 74% and 74%, respectively, in WP, G-5 and SH media. The SH medium produced the lowest shoot length and root length which were significantly lower than the MS, WPM and G-5 media. These data show in Table 45 that initial incubation medium does effect germination. From the results, it would appear that MS, WP and G-5 media were not superior to each other in terms of their ability to produce shoots and roots. However, using the results presented in Table 45, it would be difficult to exploit the culture medium for SE germination. Therefore, MS, WP or G-5 media would be tested as capable of normal SE germination.

4.1.6.4 : Carbohydrate source

The effect of different sugars (sucrose, glucose and fructose) upon zygotic embryo development was investigated. To do so, the MS medium was supplemented with 4% of all tested sugars. Additionally an experiment was done to test the effects of five concentrations of sucrose (2, 4, 6, 8, 10%).

The influence of various carbohydrate sources on the germination of zygotic embryos is shown in Table 46a. The frequency of germinated embryos was influenced by the carbohydrate sources used. The germination frequency rate with sucrose (74%) was after 14 days culture was lower than the percentage of embryos germinated on glucose (80%). No embryos developed with healthy-looking plantlets in the control treatment. However, the embryos were then transferred to the standard incubation conditions for another 2 weeks, after which time they were examined. All of them had germinated. This shows that the development of zygotic embryo cultures and standard conditions depends on sugars. In terms of shoot length there was no significant difference among the tested carbohydrates. However, glucose produced significantly longer roots than fructose.

Table 46a : Effects of sugars on germination of zygotic embryos of *P. vera*.

Treatments	% of germinated embryos	Mean shoot length (mm) ± SE	Mean root length (cm) ± SE
Control	0.0	-	-
Sucrose	74	17.72 ± 1.51 a	43.45 ± 7.91 ab
Glucose	80	14.91 ± 1.98 a	51.16 ± 6.86 b
Fructose	74	16.54 ± 2.56 a	34.09 ± 5.49 a

Data recorded on the 14 th day of incubation represents an average of 15 replicates per treatment. Means in a column followed by the same letters are not significantly different at p = 0.05 according to the Student t-test.

Table 46b : Effect of sucrose concentration on germination of zygotic embryos of *P. vera*.

Treatments	% of germinated embryos	Mean shoot length (mm) ± SE	Mean root length (cm) ± SE
2 %	74	6.900 ± 0.57 a	21.00 ± 2.14 a
4 %	74	10.63 ± 1.07 b	34.09 ± 5.49 b
6 %	67	10.30 ± 1.20 b	38.10 ± 3.63 b
8 %	74	10.27 ± 1.12 b	45.36 ± 4.21 bc
10 %	74	13.63 ± 1.33 c	56.18 ± 5.80 c

Data recorded on the 14 th day of incubation and presents an average of 15 replicates per treatment. Means in a column followed by the same letters are not significantly different at p = 0.05 according to the Student t-test.

The effects of various concentrations of sucrose on zygotic embryo germination is presented in Table 46b. There were no significant differences in the frequencies of the germinating embryos between applied treatments (Table 46b, $P > 0.05$). 14 days after culture 74%, 74%, 67%, 74% and 74% of cultured embryos germinated on the MS medium supplemented with 2, 4, 6, 8 and 10% sucrose in turn. With increased sucrose the root length increased. According to quantitative evaluation of the germinated zygotic embryos the longest shoot length and root length occurred only on the 10% sucrose treatment.

On the basis of these results sucrose may be selected as a primary carbohydrate source and subsequently various sucrose concentrations may be investigated in the sequence of the culture steps. From these results it may also be suggested that healthy embryo germination depends on the carbohydrate type and concentrations, because the carbohydrate sources tested in the MS medium had a pronounced effect on shoot length and root length (Table 46a and b).

4.1.6.5 : Effect of BAP and K on zygotic embryo germination

Plant growth regulators tested were cytokinins such as BAP and Kin, auxins such as NAA and 2,4-D each at 2 mgl^{-1}.

Treatments with auxins induced a tendency to callus formation and inhibition of healthy shoot formation. Therefore the results were not presented here. The effects of various cytokinins on zygotic embryo germination are presented in Table 47. The auxins applied inhibited germination of zygotic embryos and started to produce callus from radicle tips 3 days after culturing. The only healthy radicle and shoot elongation occurred upon transfer onto a germination medium that did not contain growth regulators.

Table 47 : Effects of growth regulators (BAP and Kin) on germination of zygotic embryos of pistachio.

Plant growth regulators (2 mgl^{-1})	% of germinated embryos	Mean root length (cm) ± SE	Mean shoot length (mm) ± SE
Control	87	28.92 ± 2.19 a	21.15 ± 1.15 a
BAP	67	25.10 ± 2.85 ab	13.00 ± 0.88 b
Kin	74	23.81 ± 1.61 b	10.27 ± 0.81 b

Data recorded on the 14 th day of incubation and presents an average of 15 replicates per treatment. Means in a column followed by the same letters are not significantly different at p = 0.05 according to the Student t-test.

There was no significant difference in the frequencies of the germinated embryos in the results presented in Table 47 between the treatments. However, the control treatment gave the highest frequency (87%) of normally germinated embryos. Treatments with cytokinins also tended to inhibit root formation but generally the embryos germinated. Depending on the types of growth regulators used, delayed development, complete inhibition or partial inhibition of embryo development occurred in all treatments where growth substances were applied. For example, when zygotic embryos of pistachio were cultured on a medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) root formation was completely inhibited and shoot elongation and induced callus from the radicle tips. From the experiment it appears that growth substances had a inhibiting effect on the healthy germination of zygotic embryos of pistachio. Along with this,

it may be suggested that both auxins and cytokinins may inhibit or stimulate partially or complete germination of SEs of pistachio. Therefore a detailed study of both auxins and cytokinins on SE germination will be undertaken.

4.1.6.6 : Effect ABA on zygotic embryo germination

ABA was tested at 0.25 mgl^{-1}, 1.0 mgl^{-1} and 2.0 mgl^{-1}. All cultures were incubated under conditions described in section 4.1.6.

The effect of ABA on zygotic embryo germination are presented in Table 48. There was significant difference in the frequencies of the germinated embryos in the presented results in Table 47 between the applied treatments. Germination was greatest with the lowest ABA treatment (0.5 mgl^{-1}). The 0.25 mgl^{-1} ABA treatment produced the highest shoot length and root length which were significantly higher than 1 and 2 mgl^{-1} ABA treatments. Root elongation was generally inhibited in the 2 mgl^{-1} ABA treatment. Interestingly, when the embryos in the 2 mgl^{-1} ABA treatment were transferred on the growth regulator-free medium, the majority of the embryos produced elongated roots. This indicates that ABA concentration may play a significant role in the inhibition of precocious germination of SEs.

Table 48 : Effects of ABA on germination of zygotic embryos of pistachio.

Treatments ABA (mgl^{-1})	% of germinated embryos	Mean shoot length (mm) ± SE	Mean root length (cm) ± SE
0.25	87	11.76 ± 1.15 a	27.53 ± 2.85 a
1.00	60	8.660 ± 0.52 b	16.88 ± 1.30 b
2.00	34	8.000 ± 1.00 b	3.00 ± 0.700 c

Data recorded on the 14 th day of incubation and represents an average of 15 replicates per treatment. Means in a column followed by the same letters are not significantly different at p = 0.05 according to the Student t-test.

4.1.6.7 : Effect of light on zygotic embryo germination

The effect of light on zygotic embryo germination was investigated by incubating isolated embryos in complete darkness and in continuous light (40 $\mu mol\ m^{-2}\ s^{-1}$).

According to quantitative evaluation of the germinated zygotic embryos, light had no effect on shoot length and root length but leaves produced in complete darkness were very thin and unhealthy (Table 49). There was no significant difference in the frequencies of the germinated embryos in the presented results in Table 49 between the treatments applied. The inhibitory

effect of the darkness on shoot colour and leaf size reversal of these effects by light suggests that phytochrome may be involved in germination.

Table 49 : Effect of light on germination of zygotic embryos of *P. vera*.

Treatments	% of germinated embryos	Mean shoot length (mm) ± SE	Mean root length (cm) ± SE
Continued light	74	10.00 ± 0.72 a	32.63 ± 4.08 a
Complete darkness	80	10.08 ± 0.97 a	32.41 ± 3.60 a

Data were recorded on the 14 th day of incubation and represent means of 15 replicates per treatment. Means in a column followed by the same letters are not significantly different at p = 0.05 according to the Student t-test.

4.1.6.8 : General consideration

Using solid media, germination of extracted axenic embryos was significantly better than whole kernels covered with testa. The average germination of extracted embryos on the agar-solidified medium without plant growth regulator was approximately 90% but less than 50% of whole kernels germinated (data not presented). However, when the testa was removed from the kernel, the growing plantlets were more vigorous than the isolated embryos. This has significant implications for the interpretation of seed storage protein studies. From all of the above experiments it can be concluded that germination may be speeded up by the different treatments i.e. by incubating embryos under different culture conditions. A growth regulator-free medium could be used for the germination of SEs. However a population of zygotic embryos is not uniform. It is not possible to predict these conditions suitable for SE germination. Therefore all of those factors must be determined empirically for the germination of SEs. Overall, the above experiments have established some of the basic characteristics of the growth forms of a primary explant system, the excised embryos of *P. vera*. The knowledge obtained on germination and development of the zygotic embryos will be considered in a detailed investigation into the germination of SEs in the following subsection.

4.1.7 : GERMINATION OF SOMATIC EMBRYOS

4.1.7.1 : Effects of growth substances on the germination of somatic embryos

After 4 weeks of maturation on MS medium supplemented with 2 mgl^{-1} BAP, individually selected embryos (20 SE per treatment) were cultured on a MS medium supplemented with abscisic acid (ABA), BAP and K each at 1 and 2 mgl^{-1}, 4% sucrose 500 mgl^{-1} casein hydrolysate, 50 mgl^{-1} l-ascorbic acid and 0.7% agar together with a control treatment for their ability to affect

germination of SE. Forty days after the culture, the number of germinated SEs were counted and morphological characteristics were recorded.

After forty days of culture, all tested treatments produced germinated embryos (Table 50). Use of BAP or K-containing media at both concentrations also gave a positive response on embryo germination. The best results were obtained when the embryos were cultured on growth regulator-free MS medium. However, there was no evidence of a significant difference in the frequencies of germinated SEs between the tested treatments (Table 50). When the germination medium was supplemented with 2 mgl^{-1} of ABA there was a pronounced negative effect on germination. Only 20% of the SEs germinated and the remainder swelled or recallused. A few non-responsive SEs on the culture were also observed.

Table 50 : Effects of different concentrations of ABA, BAP, Kin and growth regulator-free MS medium on the germination of SE*.

Growth substances	Concentrations (mgl^{-1})	Germination (%)**
Control	-	60
ABA	1	55
	2	20
BAP	1	60
	2	55
Kin	1	55
	2	50
χ^2 test		N.S.***

**Before transfer of mature SE to MS solid germination medium, SEs were matured on agar-solidified MS medium supplemented with 2 mgl^{-1} BAP. **Results are from a single experiment consisting of 20 SEs per treatment. ***Indicates that there is no significant difference.*

Most of the cultured embryos produced roots but shoots did not elongate. In addition, SEs germinated most vigorously when incubated in PGR-free medium. These results demonstrate that germination of SEs could be obtained in PGR-free medium. In the following section, germination of clusters of SEs in a growth regulator-free medium is investigated in more detail.

4.1.7.2 : Effects of ABA, BAP and sucrose during maturation on the germination of somatic embryo

To determine the effects of ABA, BAP and sucrose treatments during maturation on the subsequent germination of SE, clusters of mature SEs (Fig. 23) matured in the section 4.1.5.6 were transferred from the liquid onto the surface of 0.7% agar-solidified MS medium supplemented with 4% sucrose 500 mgl^{-1} casein hydrolysate and 50 mgl^{-1} l-ascorbic acid for germination. Thirty SEs were used in each treatment, and each experiment was repeated once.

SEs were considered to have germinated as soon as radicle elongation was observed. The numbers of germinated SEs was counted after 10, 20, 30 and 40 days of culture. A logistic regression analysis using a generalised linear model was fitted to analyse the data sets.

Table 51 : Analysis of deviance on the germination of SEs.

Change	df	Deviance	Mean deviance	Deviance ratio
+ treatment	1	116.56	116.56	91.79
+ day[1]	3	1182.98	394.33	310.53
+ growth*regs.[2]	3	871.29	290.43	228.71
+ week[3]	1	267.82	267.82	210.91
+ day*growth regs.[4]	9	59.05	6.56	5.17
+ rep[5]	1	23.64	23.64	18.62
+ suc[6]	3	8.61	2.87	2.26
+ rep*suc[7]	3	11.14	3.72	2.93
Residual	487	618.41	1.27	
Total	511	3159.51	6.18	

[1]The duration of the culture for germination (10, 20, 30 or 40 day). [2]Indicates BAP or ABA [3]The duration of the maturation (2 and 3 weeks).[4]Indicates the interaction between the duration of the culture for germination and growth regulators. [5]Indicates replication. [6]Indicates sucrose level. [7]Indicates the interaction between replication and sucrose.

Table 52 : Fitted probabilities (P) of the germinated SEs by week, day, growth regulators and sucrose.

	Treatments						
		BAP			ABA		
	Rep	1	2	Overall	1	2	Overall
Week*	2	0.58	0.54	0.56	0.49	0.45	0.47
	3	0.46	0.42	0.44	0.37	0.34	0.36
Day**	10	0.32	0.28	0.30	0.24	0.21	0.23
	20	0.47	0.43	0.45	0.38	0.34	0.36
	30	0.60	0.56	0.58	0.51	0.47	0.49
	40	0.68	0.64	0.66	0.59	0.56	0.58
Growth regulators (mgl^{-1})	0.0	0.64	0.60	0.62	0.55	0.52	0.54
	0.5	0.60	0.57	0.59	0.51	0.48	0.50
	1.0	0.48	0.44	0.46	0.39	0.35	0.37
	2.0	0.35	0.31	0.33	0.27	0.24	0.26
Sucrose (% w/v)	2	0.55	0.48	0.52	0.46	0.39	0.43
	4	0.53	0.49	0.52	0.44	0.40	0.42
	6	0.51	0.47	0.49	0.42	0.39	0.41
	8	0.49	0.49	0.49	0.40	0.40	0.40

**The duration of the maturation. **The duration of the culture for germination.*

All the main effects and interactions were very highly significant ($P < 0.001$). The conclusions from the analysis of deviance (Table 51) can be adequately interpreted in terms of the fitted values for the germination probabilities. Such fitted values are shown in Table 52. As can be seen from Table 51 the overall fit of the linear model is quite adequate as the mean residual deviance is about unity. The overall probability of obtaining germinated SEs was found to be

0.46. In general, the germination rate was lower for SE maturated on ABA medium than for those maturated on BAP medium and the first replication gave a higher rate than the second one. There was also a significant difference between the growth substances and between the treatments tested. As can be seen from Table 52, there is a steady decrease in the probability of SE germination with an increase in the concentration of growth hormone used for SE maturation (BAP or ABA).

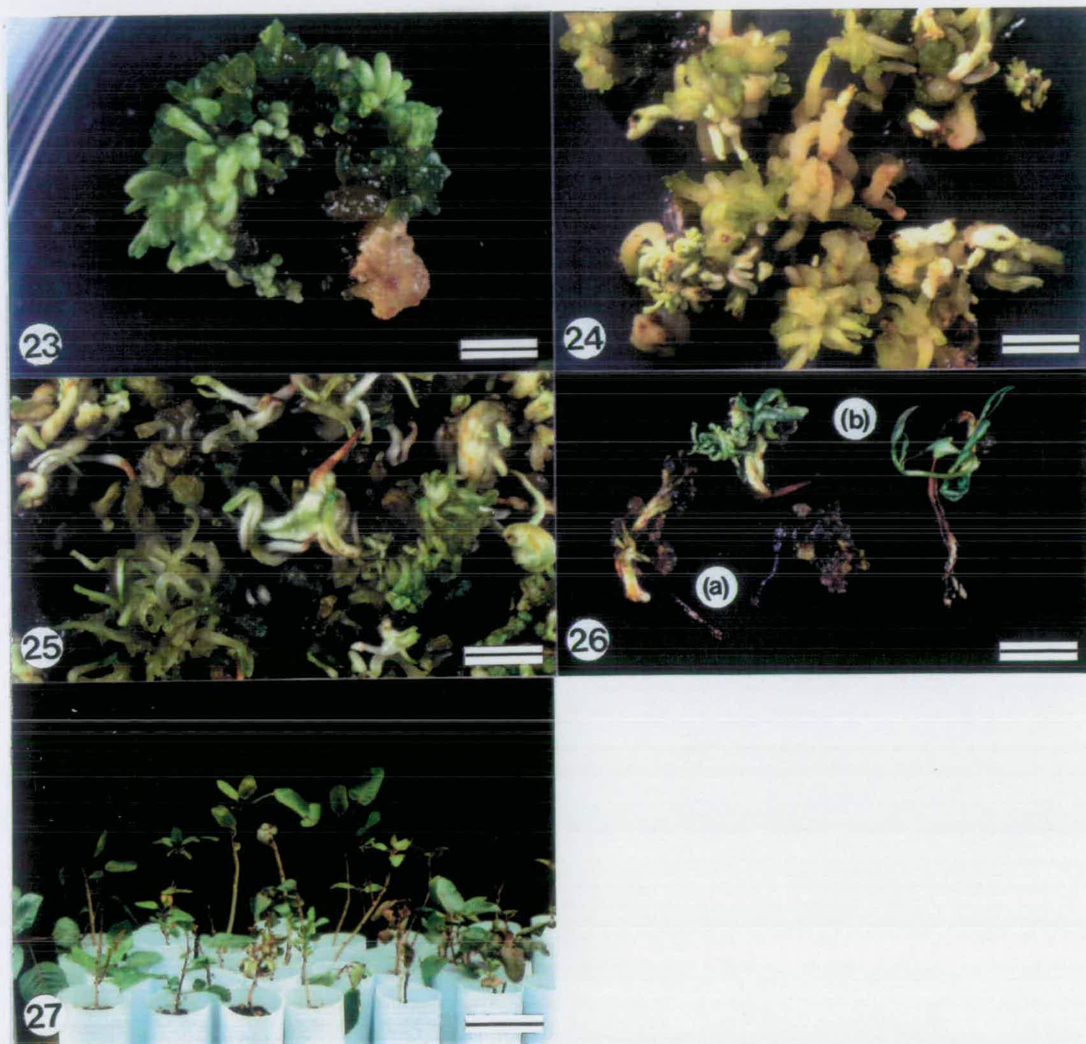


*Fig. 23 : Clusters of fully mature SEs after 2 week maturation on MS medium containing 0.5 mgl^{-1} BAP (10 days after on MS germination medium containing 4% sucrose), bar = 6 mm, **Fig. 24** : Callusing SEs (after 3 week maturation on liquid MS medium in the presence of 2 mgl^{-1} ABA) 30 days after on MS germination medium containing 4% sucrose, bar = 9 mm. **Fig. 25** : Germinated SEs (after 2 week maturation on liquid MS medium in the absence of PGR) after 40 days of culture in germination medium containing 4% sucrose, bar = 9 mm. **Fig. 26** : Undeveloped (a) and developed (b) emblings 40 days after on MS germination medium (matured in 1 mgl^{-1} ABA, bar = 10 mm. **Fig. 27** : Emblings originated from somatic embryos 6 months after transplanting, bar = 83 mm.*

The frequency of germination (0.62) was highest for the PGR-free control treatment and lowest for 2 mgl^{-1} ABA or BAP, 0.33 and 0.25 respectively). Prolongation of the maturation period from 2 to 3 weeks decreased the probability of germination in both growth substances. Germination probabilities varied from 0.56 to 0.44 and 0.48 to 0.35 for BAP and ABA, respectively.

There was a significant effect of the duration of the culture on germination. Slower and lower germination rates were mostly observed with 3-week matured SEs with 2 mgl^{-1} ABA. The probabilities of obtaining germinated SEs generally increase with an increase in the duration of culture, and reached the highest probabilities (0.66 and 0.58 for BAP and ABA, respectively) after 40 days. The concentration of sucrose in the maturation medium had a significant effect on germination, and the highest probabilities of germinated embryos were obtained with 2% sucrose (0.52 and 0.43 for BAP and ABA, respectively). However, there was no obvious pattern for the probability of germination between the concentrations of sucrose. All of the tested maturation treatments with or without ABA and BAP promoted germination of SEs. There was no inhibition of SE in media without growth substances but the maturation media applied especially 2 mgl^{-1} ABA inhibited germination of some mature SEs and produced callusing tissues (Fig. 24). The majority of the SEs showed elongated radicles within 40 days (Fig. 25). SEs matured for 3 weeks in the shaking flasks were dark green in colour.

4.1.8 : Plantlet development on the germination medium

To determine the effects of ABA, BAP and sucrose treatments during maturation on the subsequent germination and plantlet development of SEs matured in the section 4.1.5.6 were transferred from the liquid onto the surface of 0.7% agar-solidified MS medium supplemented with 4% sucrose 500 mgl^{-1} casein hydrolysate and 50 mgl^{-1} l-ascorbic acid for germination. Thirty SEs were used in each treatment, and each experiment was repeated once. SEs were considered to have germinated as soon as radicle elongation was observed. Germinated SEs (section 4.1.7) developed into plantlets on the germination medium. As soon as a germinating SE elongated its epicotyl, it was considered to be a plantlet. The number of plantlets was determined after 10, 20, 30 and 40 days of culture on the germination medium. A logistic regression analysis using a generalised linear model was fitted to analyse the data sets.

Within 40 days, most of the SEs were recallused or swollen in the treatments with high concentrations of ABA (Fig. 26). Development of the epicotyl took place after 3 weeks of culture under germination conditions. Only germinated SEs that developed epicotyls were counted as plantlets. Cotyledons grew better in the control treatments. Some developing plantlets matured in the 1 or 2 mgl^{-1} BAP or ABA were overgrown by recallusing, and hypocotyls showed

malformations. Single radicle elongation was observed in the majority of the developing plantlets but shoot elongation was usually poor in all tested treatments.

Only germinated SEs that developed epicotyls were counted as plantlets. Table 53 gives the analysis of deviance on the plantlet development of SEs. The main and interaction effects were fitted in the order in which they occur in this table. Among the main effects, duration of culture for plantlet development (day), growth substances (ABA and BAP), the duration of the maturation (week) and sucrose, all the interactions were very highly significant ($P < 0.001$). Fitted probabilities of plantlet development are illustrated in Table 54. The probability of plantlet development remained almost the same for the different replications. As in the germination frequencies, the fitted probabilities varied significantly with the concentrations of growth regulator used. It can be seen from Table 54 that germinated SEs were almost twice as likely to develop into plantlets when the cytokinin BAP was used for maturation. The number of plantlets recovered was significantly affected by the ABA and BAP concentrations, and the probabilities ranged from 0.24 to 0.13, and from 0.45 to 0.27, respectively.

Table 53 : Analysis of deviance on the plantlet development of SEs.

Change	df	Deviance	Mean deviance	Deviance ratio
+ treatment	1	622.73	622.73	385.97
+ day[1]	3	537.33	179.11	111.01
+ growth regs.[2]	3	511.14	170.38	105.60
+ week[3]	1	126.42	126.42	78.30
+ week*growth regs.[4]	3	23.98	7.99	4.95
+ day*growth regs.[5]	9	30.31	3.37	2.09
+ suc[6]	3	13.38	4.46	2.76
+ rep[7]	1	4.37	4.37	2.72
Residual	487	785.73	1.61	
Total	511	2655.40	5.20	

[1]The duration of the culture for plantlet development (10, 20, 30 or 40 day). [2]Indicates BAP or ABA. [3]The duration of the maturation (2 and 3 weeks).[4]Indicates the interaction between the duration of the culture for plantlet development and growth regulators. [5]Indicates the interaction between the duration of the culture for plantlet development and growth regulators. [6]Indicates sucrose level. [7]Indicates replication.

However, the probability of plantlet recovery was the highest in the control treatment (growth regulator-free medium). The prolongation of the maturation period from 2 weeks to 3 weeks had a negative effect on the number of plantlets obtained. The fitted probabilities varied significantly with the duration of the maturation and the higher probabilities of 0.44 and 0.24 were obtained with BAP or ABA, respectively. There was a substantial increase in the probabilities with the duration of culture for plantlet development (day) throughout the tested range. The prolongation of the duration of the culture from 10 to 40 days resulted in an increase in the number of

plantlets obtained (0.25, 0.37, 0.46 and 0.50, and 0.11, 0.18, 0.25 and 0.29 for BAP and ABA, respectively). The concentration of sucrose in the maturation medium showed no obvious pattern. In this study, after 40 days of culture it is readily apparent that the optimal concentrations of growth substances and sucrose for obtaining well-developed plantlets were 0.5 mgl^{-1} BAP with 4 or 6% sucrose with 2 week matured SEs. However, the highest frequency was obtained on the control medium without any growth regulator.

Table 54 : Fitted probabilities (P) of plantlet development from SEs by week, day, growth regulators and sucrose.

		Treatments					
		BAP			ABA		
	Rep	1	2	Overall	1	2	Overall
Week**	2	0.45	0.44	0.44	0.25	0.24	0.24
	3	0.36	0.34	0.35	0.19	0.18	0.18
Day*	10	0.26	0.24	0.25	0.12	0.11	0.11
	20	0.38	0.36	0.37	0.19	0.18	0.18
	30	0.47	0.45	0.46	0.26	0.25	0.25
	40	0.51	0.50	0.50	0.30	0.29	0.29
Growth regulators (mgl^{-1})	0.0	0.53	0.51	0.52	0.31	0.30	0.30
	0.5	0.46	0.44	0.45	0.25	0.24	0.24
	1.0	0.36	0.34	0.35	0.18	0.17	0.17
	2.0	0.28	0.27	0.27	0.13	0.12	0.13
Sucrose (% w/v)	2	0.38	0.37	0.37	0.20	0.19	0.19
	4	0.42	0.40	0.41	0.23	0.22	0.22
	6	0.42	0.40	0.41	0.23	0.22	0.22
	8	0.41	0.39	0.40	0.22	0.21	0.21

**The duration of the culture for plantlet development. **The duration of the maturation.*

4.1.9 : Weaning and growth of plantlets in soil substrate

Plantlets developed *in vitro* from SEs were washed overnight under running water and potted in a mixture of peat and perlite (1:1, v/v) or peat and grit (1:1, v/v). Plantlets were weaned in two stages first at 90±5% RH for 2 weeks and then at 70±5% RH for 2 weeks before transfer to normal greenhouse conditions (65-80% RH). Nutrient feed (Salinure 7) was provided to plants undergoing weaning on a regular weekly basis.

Survival of plantlets improved when they remained under high humidity for several weeks before transfer to greenhouse conditions. A two-week period of weaning by progressive reduction of humidities from 90% to 65% RH was beneficial in order to obtain higher plantlet survival rates. Under the acclimatisation conditions, the survival of plantlets was much higher in the peat-grit mixture than in the peat-perlite mixture. In the peat and grit, plantlets tended to resume shoot growth very quickly. Fungal contamination of the substrate was occasionally observed. Plantlets with at least two pairs of new leaves reached 5-7 cm in the mixture of peat and grit in 4 weeks and there were at least two pairs of new leaves on each plant. I observed a variation in the

number of plantlets that could be transplanted depending on the maturation treatments. The most vigorous plantlets were those that matured for 2 weeks on liquid media devoid of growth regulators or 0.5-2 mgl^{-1} BAP and 0.5 ABA (70, 60, and 60%, respectively). The lowest number of plantlets transferred (20%) was from the 3 week maturation treatment with 1 and 2 mgl^{-1} ABA, whereas they were 35 and 20%. The entire process, from maturation to obtaining plantlets in the greenhouse, required 10 to 12 weeks (4 weeks of acclimatisation). Under the method studied in this section, I have established in soil over 200 plantlets of pistachio. Developing plantlets regenerated via organogenesis or embryogenesis showed a similar pattern of growth in the greenhouse. In the greenhouse the majority of the plantlets became dormant and did not set new terminal buds. Acclimatised plantlets resumed growth after transfer to a soil and grit mixture, and developed to maturity (Fig. 27). Only 20% (40 out of 200) of them managed to survive after 3 months in the greenhouse.

4.1.10 : PLANT REGENERATION FROM ENCAPSULATED EMBRYOIDS AND AN EMBRYOGENIC MASS OF PISTACHIO, *P. VERA* L.

The aim of the present investigation was to retain the viability of EMS and somatic embryos after encapsulation and storage in an alginate matrix and to determine whether the encapsulated embryos can withstand desiccation. Since EMS cultures consist only of densely cytoplasmic embryogenic cells and highly vacuolated cells, the dry matter in these cultures may be directly correlated to the proportion of embryogenic cells (Schuller and Reuter 1993) and is an indicator of proliferative capacity. Accordingly attention was focused on the dry matter content of encapsulated and non encapsulated EMSes in storage. The viability of the encapsulated EMS and somatic embryos was investigated immediately following encapsulation, and after storage for 60 days at 4°C.

4.1.10.1 : Embryo formation and encapsulation

Twelve days after subculture in liquid MS medium, pieces of actively growing EMS (Fig. 28) were transferred onto MS medium solidified with 0.7% (w/v) agar, supplemented with 4.0 mg l^{-1} BAP, 500 mg l^{-1} casein hydrolysate, 50 mg l^{-1} l-ascorbic acid and 4% (w/v) sucrose and incubated in the light at 25°C. Immature somatic embryos (Fig. 29) developed four weeks after incubation, and grew to maturity in a further 2 weeks on the same medium or following transfer to fresh MS medium without PGRs. The method of encapsulation was a modification of those described by Bapat and Rao (1988) and Ghosh and Sen (1994). Clusters of mature somatic embryos (Fig. 30) were isolated (Fig. 31), blotted dry on sterile filter paper for 1 min, and dipped for a few minutes into a mixture of 3% (w/v) sodium alginate in MS medium supplemented with

4% (w/v) sucrose, with or without 1.0 mg l^{-1} BAP. Each embryo, approximately 4-7 mm in length, was picked up with forceps together with 0.05-0.1 ml of alginate solution, and dropped into a 0.6% (w/v) solution of $CaCl_2.2H_2O$. The solidified drops (beads), each containing a single embryo, were kept in the $CaCl_2$ solution for 2 hours on a shaker (180 rpm) in continuous light (10 µmol m^{-2} s^{-1}). After the incubation period, the beads were recovered by decanting off the $CaCl_2$ solution and then washed three times with liquid MS medium. The resulting encapsulated embryos (Fig. 32) were then cultured in 9.0 cm diameter Petri dishes on 20 ml of MS medium solidified with agar 0.7% (w/v) and supplemented with 4% (w/v) sucrose. Non-encapsulated embryos were also cultured in the same medium as a control. Sets of encapsulated embryos (50 beads/Petri dish x 5 Petri dishes) were stored in the dark at 4°C for two months in an aseptic condition on two filter papers soaked in liquid MS medium supplemented with 4% (w/v) sucrose and 1.0 mgl^{-1} BAP. After storage the beads were cultured in 9.0 cm Petri dishes on 20 ml of MS medium solidified with agar (0.7% w/v) supplemented with 1.0 mg l^{-1} BAP and 4% (w/v) sucrose. The Petri-dishes were sealed with Parafilm and maintained in the light (25 µmol m^{-2} s^{-1}) at 25°C. Non-encapsulated-stored and non-encapsulated-unstored embryos were also cultured in the same way as a control. The chi-square (χ^2) test was used to test the differences between % of rooted embryos, % of embryos with elongating shoots and the conversion frequencies of embryos from non-encapsulated-stored, non-encapsulated-unstored, unstored-encapsulated and stored-encapsulated somatic embryos.

The percentages of rooted embryos and embryos with elongating shoots, and the conversion frequency of embryos from non-encapsulated-unstored, non-encapsulated-stored, unstored-encapsulated and stored-encapsulated somatic embryos are summarised in Table 55. No embryos developed into healthy plantlets from the non-encapsulated-stored embryos. Only 50% of the non-encapsulated-stored embryos produced roots during or after storage. None of the stored encapsulated somatic embryos showed any sign of germination during 8 weeks of storage at 4°C on MS medium with sucrose (4%) and 1.0 mg l^{-1} BAP. Within three weeks of return to culture on MS medium following storage the majority of embryos had started to produce roots which subsequently extended to establish contact with the medium. Within 4 weeks of culture, 90% of non-encapsulated-unstored somatic embryos had produced roots while 66% had developed elongating shoots and 30% had been converted into plantlets. Somatic embryos encapsulated in calcium alginate which were cultured directly onto MS medium without BAP did not show any sign of germination within the first ten days of culture, and only a few of these individuals had produced roots or elongated shoots by the end of the third week. The conversion frequency for encapsulated embryos cultured directly after encapsulation, was lower (24%) than for non-encapsulated-unstored embryos (30%) but higher than for stored-encapsulated embryos (14%). Two thirds of the encapsulated-stored embryos rooted to produce plantlets but only 26% produced shoots and 14% produced complete plantlets. This reduction in

viability could be a consequence of variable quality of the embryos used for encapsulation, although the individuals were selected carefully, but may also be attributable to the properties of calcium alginate or to stress caused by the physical handling of the encapsulated embryos (Ghosh and Sen 1994). After two months of storage at 4°C the proportion of rooted embryos varied significantly between treatments (Table 55, $P < 0.001$). The proportion of rooting was highest (90%) in non-encapsulated-unstored somatic embryos and gradually decreased to 78%, 66% and 50% respectively in unstored-encapsulated, stored-encapsulated, and non-encapsulated-stored somatic embryos. The numbers of embryos with elongating shoots was also influenced by the encapsulation treatment, those produced following storage at 4°C (26%) being significantly lower than the numbers obtained from non-encapsulated or unstored-encapsulated somatic embryos (66%, and 54%), respectively, (Table 55, $P < 0.001$). Also the conversion frequency rate for encapsulated-stored embryos (14%) was after two months of storage lower than that for non-encapsulated-unstored embryos, which showed the highest frequency of conversion (30%).

Table 55 : The frequencies of rooting, shoot elongation and conversion to plantlets of non-encapsulated-stored, non-encapsulated-unstored, unstored-encapsulated and stored-encapsulated somatic embryos.

	No. of cultured embryos*	% rooted embryos	% embryos elongating shoots	Conversion frequency (%)
Non-encapsulated-stored	50	50	0	0
Non-encapsulated-unstored	50	90	66	30
Cultured directly after encapsulation	50	78	54	24
Encapsulated-stored at 4°C for 60 days then cultured	50	66	26	14
χ^2 (3 df)		$P < 0.001$	$P < 0.001$	$P < 0.001$

**The data represent the mean of one experiment with 50 embryos per treatment.*

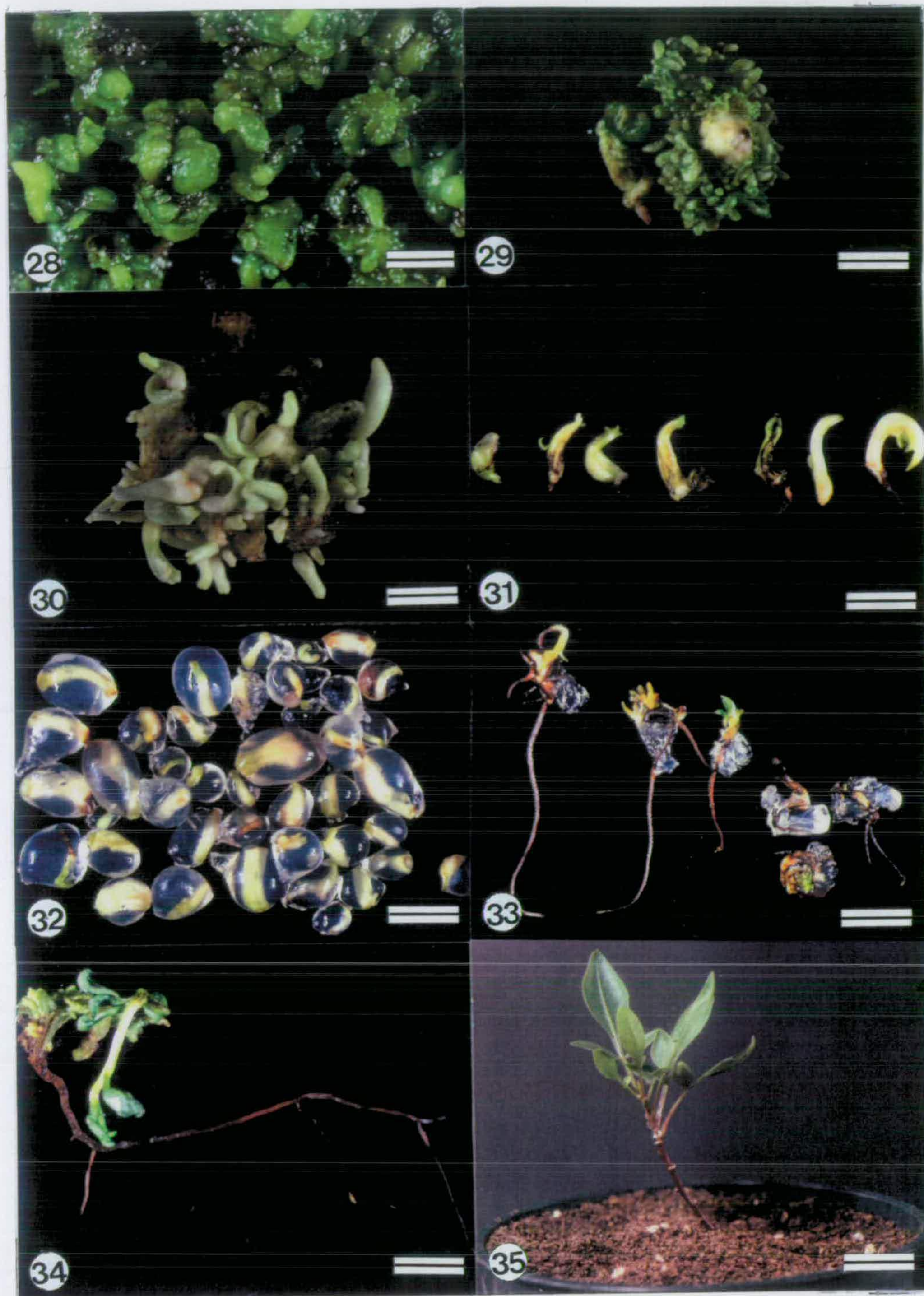
Encapsulated but unstored embryos showed an intermediate rate of conversion (24%). None of the non-encapsulated-stored embryos produced healthy looking plants. Chi-square analysis of the data showed that there were very highly significant differences ($P < 0.001$) between the frequencies of conversion to plantlets of non-encapsulated-stored, non-encapsulated unstored, encapsulated-unstored and encapsulated-stored somatic embryos following transfer to culture (Table 55).

During a typical developmental sequence, somatic embryos produced long roots within three weeks of culture before the shoots elongated (Fig. 33). Well developed plantlets (e.g. Fig. 34) were usually obtained within five weeks of culture, and when they were potted into a sterile mixture of perlite-grit (0.3-1 mm standard grades-quartzag) (1:1, v/v) compost (Fig. 35), no phenotypic or genotypic variation was observed between the plantlets.

4.1.10.2 : Encapsulation of EMS

EMSes were obtained from immature seed explants as described in section 4.1.1. Suspension cultures were started from EMSes (Fig. 28) (0.5 g wet weight) using Erlenmeyer flasks of 250 ml capacity containing 50 ml of MS medium without agar. Flasks were sealed with a double layer of aluminium foil and incubated at 25°C on an orbital shaker at 98 rpm in continuous light (25 µmol m^{-2} s^{-1}) and sub-cultured every two weeks. For encapsulation of EMS, 12-day-old cell clusters (approximately 0.5 g wet weight), growing actively on liquid MS medium without growth regulators, were washed twice in sterile distilled water, followed by a third washing with liquid MS medium. The washed EMSes were mixed with sodium alginate solution (3g fresh weight to 100 ml) for a couple of minutes. Clusters of EMS were then picked up with sterile forceps and dropped into a 0.6% (w/v) solution of $CaCl_2.2H_2O$. The encapsulated EMS beads were then shaken in the $CaCl_2$ solution for 2 hours and washed three times with liquid MS medium supplemented with 4% (w/v) sucrose and 1.0 mgl^{-1} BAP. The beads were stored aseptically at 4°C for two months in 9.0 cm Petri dishes lined with two filter papers (7.0 cm) soaked in liquid MS medium with 4% (w/v) sucrose and 1.0 mgl^{-1} BAP. Non-encapsulated-stored and non-encapsulated-unstored EMS was also cultured in the same way as a control. At the end of the storage period the alginate capsule covering the stored EMS was removed before culturing. Pieces of EMSes (0.5 g for each flask) were placed in 50 ml of MS medium in 250 ml Erlenmeyer flasks. The flasks were then sealed with a double layer of aluminium foil and placed on an shaker at 98 rpm at a light intensity of 25 µmol m^{-2} s^{-1} at 25°C. Suspension cultures were re-established by sub-culturing stored encapsulated EMS (0.5 g wet weight) into 50 ml of liquid MS medium supplemented with 8% (w/v) sucrose, with or without 1.0 mg l^{-1} BAP and sub-cultured every two weeks. For fresh weight measurements, the EMS was weighed in a 9.0 cm Petri dish. Total dry weight of the embryonic mass was obtained after 48 hours in an oven at 80°C. Each measurement is the mean of five flasks per treatment. Differences between means were tested at $\alpha = 0.05$ using Student's t-test.

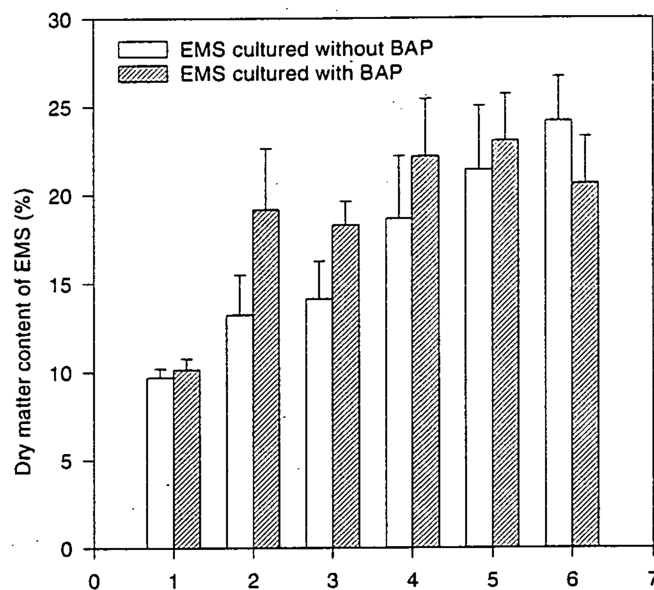
Figure 36 shows the effects of the encapsulation and storage on dry matter content of non-encapsulated-stored (Bar 1), encapsulated-stored EMSes (Bars 2 to 5) and non-encapsulated - unstored (Bar 6) EMSes. It was evident that after two months of storage at 4°C the dry matter content of non-encapsulated-stored EMS had decreased significantly (Fig. 36, Bar 1) by



***Figs. 28 to 35** : Synthetic seeds of pistachio, Pistacia vera L.*

***Fig. 28** : EMS used for encapsulation and as a source of somatic embryos. Scale bar = 13 mm. **Fig. 29** : Developing somatic embryos on MS medium supplemented with 2 mgl^{-1} BAP. Scale bar = 27 mm. **Fig. 30** : Massed somatic embryos prior to separation and selection for encapsulation. Scale bar = 36 mm. **Fig. 31** : Somatic embryos of pistachio isolated manually from EMS. Scale bar = 15 mm. **Fig. 32** : Individual Pistachio somatic embryos encapsulated in Ca-alginate beads ready for culture. Scale bar = 14 mm. **Fig. 33** : Germinated synthetic seeds on MS medium after two months of storage at 4 °C. Scale bar = 11 mm. **Fig. 34** : Pistachio plantlet derived from a synthetic seed stored for 2 months at 4 °C. Scale bar = 26 mm. **Fig. 35** : A young pistachio plant derived from a stored synthetic seed following establishment in soil. Scale bar = 8.4 mm.*

comparison with unstored EMS (Bar 6). No EMS proliferated frôm non-encapsulated-stored regenerative EMS. EMS that had been encapsulated and stored proliferated but initially had a lower dry matter content than non-encapsulated-unstored EMS. The data on bars 2 to 5 in Figure 36 show that on media without BAP the dry matter content of encapsulated-stored EMS increased with subculture number following a return to normal culture conditions. EMS that had been cultured on a medium without BAP prior to storage showed a greater dry matter loss during storage than EMS cultured with BAP. This may account for the fact that the proliferative capacity of the alginate-encapsulated EMS was initially lower than that of non-encapsulated EMS when raised on MS medium devoid of BAP (the normal maintenance medium for pistachio EMS). However, there was no difference in the dry matter content (Fig. 36) or proliferative capacity of encapsulated-stored EMS on MS medium with or without 1.0 mgl^{-1} BAP supplement after the second subculture.



Non-encapsulated-stored EMS (Bar 1), successive subcultures of encapsulated-stored EMS (Bars 2 to 5), non-encapsulated and unstored EMS (Bar 6)

Figure 36 : *The effects of the encapsulation and storage on dry matter content of EMS (%) on MS medium supplemented with 8% (w/v) sucrose, with or without 1.0 mgl^{-1} BAP. The data represent the means of two experiments with 5 replicates per treatment. The embryogenic tissue had been maintained in liquid culture for more than one year before these experiments were performed.*

4.1.11 : DISCUSSION

4.1.11.1 : Embryogenic mass initiation

In these experiments (section 4.1) all the material originated from one tree. Induction of EMS was obtained from 0%, 20%, 10% and 12.5% of the immature fruits of pistachio harvested in mid-June, mid-July, early-August and early-September, respectively (Table 34). This represents juvenility of tissues or stage of developing zygotic embryos as an important factor for the production of EMS. The difference in the frequencies of immature fruits inducing EMS may not only be due to the differences in the culture medium of the explants but also the time of year the explants were harvested (Table 34 and Onay et al. 1995a). The explants harvested in mid-June did not produce EMS because the immature fruit was not able to be isolated from the testas. An improvement could be made for the higher frequencies of EMS induction if the explant were to be cultured throughout an available season. The choice of medium was also critical for EMS initiation and none of the explants produced EMS cultured on WP medium (Onay et al. 1995a). This has not counted for the statistical analysis. Therefore, a significant relationship may exist between collection date and the medium. In addition EMS were proliferated in all treatments tested with BAP, but showed reduction of growth rate, or partial inhibition of growth, on media with the highest concentration of BAP, 16 mgl^{-1} (45 µM) In general, EMS does not require growth regulators in a culture medium to develop into SEs. These results are in agreement with results reported by Ammirato (1984). However, greyish EMS was capable of producing numerous embryos when maintained on medium containing growth regulators. These results are more typical of several species such as cassava (Shabados et al. 1987). I assume that the embryogenic capacity of the initial EMS may be unstable, but it was then conditioned by repeated subculture. These EMSes maintained their embryogenic capacity for over 15 months. These results are in agreement with results for *Raphanus sativus* reported by Jeong et al. (1995) who found that the embryogenic capacity of the initial callus is unstable, but is then stabilised by repeated subculture. Once EMS was obtained, further development of SEs may require a different stage. Somatic embryogenesis seems to have stage-specific requirements as reported by Klimaszewska (1989) for hybrid larch and by Becwar et al. (1990) for *Pinus taeda.*

4.1.11.2 : Embryo maturation

4.1.11.2.1 : Effects of carbohydrates and basal media

Variation in pistachio SE maturation (Table 40) was observed among the different carbohydrates tested. The highest frequency of SEs was obtained with sucrose in the presence of 2 mgl^{-1} BAP

and the embryos with the most normal morphology or most resembling zygotic embryos were produced in the presence of sucrose. The type of osmoticum used in conjunction with ABA profoundly affects development of conifer SEs (Attree and Fowke 1993). In *P. vera* I have reported that sucrose and glucose were the best carbohydrate source for the promotion of embryos. Coconut milk and banana powder were also completely ineffective for proliferation of EMS, maturation and germination of SEs. However, it was reported that the osmotic effect exerted by PEG was superior to the effect of sucrose at corresponding osmotic potentials (Attree et al. 1991). In addition, Roberts (1991) showed that mannitol and ABA separately had a similar effect on SE maturation in interior spruce. Likewise, the use of coconut milk in the maintenance medium has stimulated somatic embryogenesis (Button and Botha 1975). The low concentration (10% v/v) of coconut milk promoted the normal development of globular embryos (Sabharwal 1963); however, the higher concentration (40%) was harmful (Rangaswamy 1961).

The formation of high quality pistachio SEs was also dependent upon the basal media tested. Four different basal media MS, WP, SH, and G-5 were tested (Table 38). The number (measured by counting the matured) of SEs per 250 mg EMS was higher in MS medium. In treatment with MS the SEs developed normally, whereas in treatment with full strength MS medium, the effects of media became evident not only in the number of SEs developed but also the majority of the SEs had a morphology similar to the zygotic embryo remaining non-callusing. Development of SEs in *Quercus robur* cultures was stimulated on MS, WP, and SH media (Chalupa 1992 and 1993). Enhancement of embryoid production using modified MS medium supplemented with 1 mgl^{-1} BA and 1 mgl^{-1} 2,4-D has been reported for *Quercus* (Gingas and Lineberger 1989). However, the highest percentages of normal polar embryoids were produced by explants cultured on growth regulator-free MS medium. Embryogenic cultures and embryoids of *Quercus cerris* were induced on WP medium containing BA (1 mgl^{-1}), K (1 mgl^{-1}), and IBA (2 mgl^{-1}) (Ostrolucka and Pretova 1991).

4.1.11.2.2 : Effect of growth regulators

The effect of six auxins (2,4-D, NAA, IAA, IBA, Picloram and Dicamba) and cytokinins (BAP, BA, K, TDZ, 2iP, and Zea) each of them at 2 mgl^{-1} was studied (Table 41). In this study, formation of SEs was dependent on the presence or absence of auxins and cytokinins. Embryo quality and form did not benefit from the use of any of the different auxin types. All of the auxins tested, 2,4-D, NAA, IAA, IBA, Picloram and Dicamba were partially inhibitory in the maturation of embryos, and developed into swollen embryos or into soft white to yellowish callusing tissues. Cytokinin BAP was the most effective plant growth regulator for the maturation of SEs. Based on these results obtained in Table 41 BAP was chosen for further routine development of the SEs.

4.1.11.2.3 : Effects of BAP and ABA in agar-solidified MS medium on the maturation of SEs

Of the treatments tested only BAP and sucrose had significant effects on the numbers of mature SEs (Onay et al. 1995a). By week 4, on media supplemented with 4 and 8 mgl^{-1} (18 and 36 µM) BAP, increasing the sucrose concentration from 2% to 8% resulted in precocious germination of SEs. Especially in the medium supplemented with 4 mgl^{-1} BAP, half of the SEs had germinated and had elongated radicles by week 4. At 1 mgl^{-1} (4.5 µM) BAP and 2 mgl^{-1} (9 µM) BAP, precocious germination did not occur, but the white to yellowish SEs showed elongation of the hypocotyls (Onay et al. 1995a). Increasing the BAP concentration from 4 to 16 mgl^{-1} resulted in fewer mature SEs. At the lowest BAP concentrations sucrose stimulated embryo production, but above 4 mgl^{-1} BAP, further increases in the sucrose concentration above 4% had inhibitory effect, so that the highest concentrations of BAP and sucrose produced the lowest yield of SEs (Onay et al. 1995a).

In treatments with ABA (Table 42) the SEs did not germinate precociously, whereas in treatments with ABA (1.0 and 2.0 mgl^{-1}), generally the development of SEs was reduced and additional time was required for the maturation of embryos. The prevention of precocious germination is well documented in zygotic embryos (Galau et al. 1991) and has been reported for angiosperm such as alfalfa (Xu et al. 1990). By 4 week of culture, the effects of ABA and sucrose became evident only in the number of SEs developed and most importantly in the morphological features. On media supplemented with 2 mgl^{-1} ABA (Table 42), the decrease of the sucrose concentration from 8% to 2% resulted in an inhibition of SE development. However, in the presence of 2 mgl^{-1} ABA with higher levels of sucrose (6% and 8%), in some plates SEs were also developed and they were grown, had elongated hypocotly and cotyledons after an additional 2 weeks of maturation time. In contrast, SEs obtained on media with BAP treatments had started radicle growth during the 4 weeks of culture (Onay et al. 1995c). In addition this reduction was also seen when a low level of ABA (0.25) was combined with different levels of sucrose because there were only minor differences in the number of SEs produced at 2%, 4%, 6% and 8% sucrose combined with 0.25 mgl^{-1} ABA. In general the mid levels of ABA (0.5 and 1 mgl^{-1}) and increasing the sucrose concentration were favourable for a high frequency of matured SEs, whereas at 0.5 ABA an increase in sucrose concentration from 2% to 8% reduced the number of SEs. Abnormal SE development in carrot (Ammirato 1983) and interior spruce (Roberts et al. 1990) is known to be suppressed by ABA. Enhancement of SE production using ABA has been reported for interior spruce (Roberts et al. 1990) and black spruce (Cheliak and Klimaszewska (1991). The sucrose and ABA or BAP interaction, statistically significant in my study, was also found in some conifer species (Hakman and von Arnold 1988, Finer et al. 1989 and Cheliak and Klimaszewska 1991) but the development of SEs was partially inhibited by the use more than 0.5 mgl^{-1} ABA.

4.1.11.2.4 : Effects of BAP and ABA in liquid MS medium on the maturation of SEs

To investigate the effects of BAP, ABA and sucrose concentrations during maturation in liquid MS medium on germination frequency and plantlet development in Pistachio SEs, the linear logistic model considered in this study is constructed by writing the logit of p_{ijklm} as a linear combination of all main effects, and the fitted probabilities of germination and plantlet development are presented. Of the treatments tested only BAP and sucrose had significant effects on the numbers of mature SEs. After 2 or 3 weeks on media supplemented with 0-2 mgl^{-1} BAP (Table 43), increasing the sucrose concentration from 2% to 8% did not result in precocious germination of SEs. None of the SEs had germinated and had elongated radicles by week 2. The white to yellowish SEs showed elongation of the hypocotyls. Increasing the BAP concentration from 0.5 to 2 mgl^{-1} resulted in fewer mature SEs.

Of the treatments tested only ABA and sucrose had significant effects on the numbers of mature Ses (Table 44). In treatments with ABA the SEs also did not germinate precociously. The prevention of germination has been reported for pistachio zygotic embryos as reported in section 4.1.6. By 2 week of culture, the effects of ABA and sucrose became evident in the number of SEs. On media supplemented with 2 mgl^{-1} ABA, the increase of the sucrose concentration from 2% to 8% resulted in inhibition of SE development. However, in the presence of 2 mgl^{-1} ABA SEs were also developed when they were kept in the culture for an additional week of maturation time.

Pistachio SEs can be matured in liquid or agar-solidified medium with or without ABA and BAP. Successful maturation of SEs was achieved after culture for two weeks in liquid MS medium. Development of SEs using a liquid culture maturation phase, e.g., *Picea abies* (Boulay et al 1988), *Picea glauca* (Attree et al. 1994), *Picea mariana* (Tautorus, 1990), *Picea sitchensis* (Krogstrup, 1988), and *Pseudotsuga menziesii* (Durzan and Gupta 1987) has also been reported. Cultures maintained on agar-solidified medium produced SEs as much as liquid medium Media with 4 mgl^{-1} (17.8 µM) BAP and 4% sucrose induced most embryos (49.2 per 250 mg fresh weight) (Onay et al. 1995a). These results suggest that maturation may be achieved in liquid or agar-solidified media but maturation on agar-solidified was longer than in liquid medium, and germination and plantlet development was highest when cultures had been grown in liquid medium. I assume that the poor performance of material maintained on agar-solidified medium reflected the relative connection of embryos that had been cultured in agar-solidified medium, and the developing SEs in liquid medium were able to drain a greater source of nutrients. The benefits of the liquid culture system include saving time and labour.

The maturation of SEs in pistachio depends on a number of factors and their interactions. Studies in section 4.1.5 revealed all of them and, more importantly, the interactions among them (sucrose concentration and ABA or BAP concentration). In addition the maturation of SEs was dependent on the origin of EMSes, because the EMS induced from leaf tissues did not promote the developing embryos without BAP, but the EMS induced from immature fruit explants did promote the developing SEs in the MS medium even when devoid of any growth regulators. Therefore, to achieve the most efficient maturation of SEs we have to combine all these factors in order to fully exploit the embryogenic potential of pistachio varieties.

4.1.11.3 : Effects of BAP and ABA on germination and plantlet development of SEs

Development of plantlets from SEs of pistachio was affected by many factors. The most important factors influencing germination and plantlet growth in pistachio were the time that the SEs were left on the germination medium, and the concentrations of growth regulators used. The maturation treatment of control gave rise to developmentally highest frequency of germinating (62%) and developing plantlets. I assume that this may be the right culture conditions for the right tissue. There was not an adverse effect of the duration of the maturation on germination and plantlet development. However, the increase in the duration of the ABA or BAP maturation period on agar-solidified medium led to a decrease of the SEs conversion rate (Onay et al. 1995c). In numerous other species the level of endogenous ABA prevented precocious germination of the embryos (Xu et al. 1990). These are in agreement with my results that did not show precocious germination. In this study, the highest germination frequency was obtained with SEs matured in the absence of ABA or BAP. This is not in agreement with the results reported by Roberts et al. (1990) for somatic embryos of interior spruce.

Germination and plantlet recovery were found to be reliable indicators of the optimal or suboptimal maturation treatments of pistachio SEs. The highest germination frequency was obtained when 2 weeks maturation treatments were applied without plant growth regulators. When the rapidity of germination was evaluated, the three weeks maturation treatment was deleterious. The treatment of 2 weeks seemed more beneficial than 3 weeks without plant growth regulators. Morphological changes of the SEs were observed during the third week of maturation; they enlarged and turned dark green. I assume that the embryos become dormant because of the physical and chemical environment. This could explain the lower germination of the SEs matured for 3 weeks in liquid medium. The cultures were kept for 4 weeks in liquid medium, then transferred to a germination medium few SEs germinated. As with germination frequency, plantlet recovery was affected by maturation time, with 2 weeks being superior respective of the ABA or BAP concentration used.

The overall probability of plantlet development from an embryo, 0.36 was lower than that of the control treatment (0.52) when the cytokinin BAP was used for maturation (Table 54). All main factors, BAP, week, sucrose and day, have significantly affected the plantlet development when the cytokinin BAP was used for plantlet development. There was evidence of a significant difference in the probabilities for the first and last duration's of the culture. The level 0.5 mgl^{-1} of BAP gave the highest probability of plantlet development, 0.45. The highest probability was obtained with the concentrations of 4-6% (w/v) of sucrose. The plantlet development frequencies decreased gradually with the duration of maturation (week) (Table 54). The optimum concentrations of main factors giving the highest probability of plantlet development were obtained with 0.5 mgl^{-1} BAP together with 4 or 6% (w/v) sucrose concentration after 40 days of culture and a 2 week maturation period in liquid MS medium. However, the optimum concentrations of main factors giving the highest probability of plantlet development were obtained with 2 mgl^{-1} BAP together with 8% (w/v) sucrose concentration after 14 or 21 days of culture and 6 weeks of maturation period on agar-solidified medium (Onay et al. 1995c). Therefore, using the liquid MS medium for the maturation of SEs produced not only a recovery of plantlets but also reduced time for the regeneration of plantlets. The overall probability of plantlet development from an embryo was rather low, 0.18 when ABA was used for maturation (Table 54). The optimum levels of main factors giving the highest probability of plantlet development were obtained with 0.5 mgl^{-1} ABA together with 4%, 6%, and 8% sucrose concentrations after 40 days of culture and a 2-week maturation period. Apart from maturation period, similar optimum levels of main factors were obtained when ABA was used for maturation in agar-solidified MS medium (Onay et al. 1995c). Morphological changes of the SEs were observed during the culture period. In particular, embryos matured in BAP (0.5-2 mgl^{-1}) or more 0.5 mgl^{-1} ABA treatments swelled and re-callused from the root tips. Extensive cotyledon enlargement was observed generally on embryos matured in the control and BAP treatments. For pistachio, BAP has the capacity to induce EMS and embryo formation on immature fruit explants, but BAP has not been widely used for SE maturation and plantlet development in other plant species.

In this study, ABA or BAP were added only during maturation and EMSes were maintained in liquid medium without growth regulators. Neither germination nor plantlet development medium contained BAP or ABA. In fact, treatments with ABA or BAP were unnecessary for the formation of individual embryos and elongation of globular embryos because the EMSes cultured in liquid MS medium produced more SEs than BAP and ABA applied treatments. This finding is in contrast to the reports of Boulay et al. (1988) who found for *Picea abies* that treatment with ABA was necessary for the formation of individual embryos and elongation of globular embryos. With respect to plantlet recovery, the best maturation treatments were 2 weeks at 0.5 mgl^{-1} BAP or devoid of growth regulators with 4 or 6% sucrose. Therefore, with pistachio SEs, I recommend a 2 week maturation period on either 0.5 mgl^{-1} BAP or devoid of growth regulator at 4% sucrose to

generate high numbers of viable plantlets. The overall probability of a germinating embryo from a mature SE was found to be 0.46 and 0.38 when ABA or BAP, respectively, were used for maturation (Table 54). However, under the germination conditions, the overall probability of a plantlet developing from a germinated embryo was found to be 0.18 and 0.36 when the growth regulators ABA and BAP, respectively, were used for maturation in liquid MS media. The fact that the ABA controls had much lower probability than the BAP controls in sections 4.1.7.2 and 4.1.8. The reason for this could be the age of the EMS because the experiment with ABA were established 3 months after the BAP experiments.

There are few data sets on the acclimatisation of conifer plants from *in vitro* to *ex vitrum* conditions (Tautorus et al. 1991). The acclimatisation method that was developed for pistachio was easy to perform but the percentage of survival was low after transfer to greenhouse (60%). In the current study, there was also a decrease in plantlet survival three months after transfer to the greenhouse (from 60% to 20%). Acclimatised plantlet matured on agar-solidified or liquid MS medium showed a similar pattern of growth in the greenhouse. The acclimatisation of pistachio SEs matured on agar-solidified MS medium was reported by Onay et al. (1995c). This success rate is not acceptable and the technique should be improved. However, the regenerated plants may grow better under natural conditions.

4.1.11.4 : Encapsulation of EMS and embryos

The results presented in section 4.1.10 demonstrate that encapsulation of Pistachio EMS in beads of calcium alginate gel is an effective procedure for short to medium term storage of embryogenic cell lines. While there is some loss of dry matter content and proliferative capacity during storage, stored encapsulated tissue rapidly recovers full dry matter content and proliferative vigour following a return to normal culture conditions. It remains to be established whether the storage period can be extended further than the 60-day period investigated here, to facilitate longer-term tissue storage. Only a single procedure for the encapsulation and storage of embryos was tested in these experiments. Although the conversion frequency of complete plantlets from synthetic seeds of pistachio was low (14%), the results obtained were positive, demonstrating that the method is feasible, and suggesting that it may be possible to optimise these techniques to improve the rate of production of complete plantlets after storage of embryos. Both of these approaches are applicable to the preservation of desirable elite pistachio genotypes and to the management of culture stocks during production. The potential for long term storage of embryogenic cultures of *Picea glauca* (Kartha et al. 1988) and *Pinus taeda* (Gupta et al. 1987) in liquid nitrogen has been demonstrated. *Pinus caribaea* plants from cryo-stored and non-cryo-stored somatic embryos appeared similar when established in a

greenhouse (Laine et al. 1992). Conceivably, therefore, cryo-storage may also prove to be a suitable approach to long-term storage of pistachio tissues.

4.2 : SOMATIC EMBRYOGENESIS FROM CULTURED LEAF EXPLANTS OF THE PISTACHIO JUVENILE AND MATURE MATERIALS

The objective of this part of the results was to develop a true-to-type regeneration procedure. The experiments were designed to investigate the effects of juvenile (seedlings) and mature leaf explants on embryogenic callus induction and plant regeneration. For solid cultures, the explants were cultured in disposable Petri dishes (5.0 cm) containing 25 ml of MS medium, 4% sucrose, 500 mgl^{-1} casein hydrolysate, 50 mgl^{-1} l-ascorbic acid. The medium was solidified with 0.7% agar and the pH of the medium was adjusted with 0.1 N NaOH at 5.7 before autoclaving for 16 min at 121°C. For liquid cultures, 50 ml MS medium were poured into 250 ml flasks and sealed with a double layer of aluminium foil. All media containing growth regulators were autoclaved for 16 min. The cultures were kept on a gyratory shaker at 98 rmp at 25°C under continuous light (25 μ mol m^2 s^{-1}). The growth of callus from leaf explants was estimated by visual observation. Observations were recorded after 3 weeks for callus fresh weight, at the end of 90 days for the number leaves from which embryogenic callus initiated was determined for each treatment and at the end of 120, 150, 180 and 190 days for SE induction. Unless otherwise stated, in the following investigations all culture conditions were the same as above (standard conditions).

4.2.1 : Freshly germinated axenic leaf explants: callus production

The aim of the experiment was to develop a model system for the initiation of embryogenic callus, then to apply the developed method for the mature material. Therefore the following experiments were done: Seeds of pistachio were surface sterilised as explained in section 3.1.7. Then, they were cultured in order to obtain stock material. For all experiments, first pairs of fully expanded leaves from axenic shoots were excised and used as the source of explants.

4.2.1.1 : Effects of methods of incubation on callus production

The aim of this experiment was to examine the effect of methods of incubation for callus initiation from axenic leaf explants. To initiate callus, the axenic leaf explants were incubated in different ways in the agarified MS medium containing 2 mgl^{-1} 2,4-D 1 mgl^{-1} NAA, 0.5 mgl^{-1} BAP, 4% sucrose and 0.7% agar. Conditions were as follows:

A. The intact leaf was placed with the upper epidermis in contact with the medium.

B. The intact leaf was placed with the lower epidermis in contact with the medium.

C. The intact leaf with a piece of stem was placed with the upper epidermis in contact with the medium.

D. The intact leaf with a piece of stem was placed with the lower epidermis in contact with the medium.

E. The intact leaf was immersed horizontally in medium.

Alternatively, the leaf explants were initiated directly into liquid MS medium. The cultures were carried out by incubating two pairs of fully expanded leaves from axenic shoot cultures into 250 ml conical flasks of 50 ml liquid MS medium containing 1 mgl^{-1} BAP, but without agar. There were 4 replicates of each treatment, and the experiment was repeated twice. All cultures were incubated under conditions as described in section 4.2. Callus production was observed during for weeks in culture.

By the end of the 28 days, under conditions A, B and E calli were not formed even at the cut edges that were in contact with medium in places where the edges of the leaves had curled up and away from the medium. Conditions C and D resulted in little calli. There were also browning of explants that were in contact with the culture medium. This indicates that growth regulators may not be absorbed quickly through the leaf epidermis during the 4 weeks of culture. This also indicated the need for the addition of growth regulators. However, when the cultured explants were left on the same culture medium for another 4 weeks, the majority of them produced callusing tissues with very little proliferation ratios, but the conversion of explant into callusing tissues was still very low. However, all leaf explants of pistachio did produce callus or callusing tissues in liquid MS medium containing 1 mgl^{-1} BAP after four weeks of culture.

From the experiment it may be concluded that calli were not formed even at the cut edges of the many explants that were in contact with agar-solidified MS medium in places where the edges of the leaves had curled up and away from the medium during 4 weeks of culture. Moreover, little callus was produced 8 weeks after culture. In other words, the agarified induction callus required long periods of time for callusing tissues from leaf explants. However, callus initiation was strongly stimulated when liquid media were used for callus initiation. These results suggest that the use of liquid medium for the induction of callus may be more practical. Thus leaf explants that are with pedicels will be used in liquid MS medium supplemented with different growth substances for the initiation of embryogenic calli in the following investigations.

4.2.1.2 : Effects of growth regulators

To study the effects of various cytokinins on embryogenic callus induction, cytokinins such as BAP, K, Zea, 2iP, BA, TDZ were added to the medium for the culturing of leaves. Similarly, to study the effects of various auxins on embryogenesis, auxins such as 2,4-D, 2,4,5-T, dicamba,

picloram, NAA, NOA, IPA, IBA, pCPA and IAA were added to the medium used for the culturing of leaves. The ranges of concentrations of auxins and cytokinins used were 0.5, 1, 2, and 4 mgl^{-1}. The levels of TDZ were also tested at 8 mgl^{-1} 16 mgl^{-1} and 32 mgl^{-1}.

Table 56 : Effect of different cytokinin on callus morphology from leaf explants of pistachio.

Cytokinin	Concen-tration (mgl^{-1})	Quality of callus*	Callus colour
BAP	0.5	++	greyish callusing tissues
BAP	1.0	+++	greyish callusing tissues
BAP	2.0	+++	deep green callusing tissues
BAP	4.0	+++	deep green callusing tissues
BA	0.5	+	dark green
BA	1.0	+	dark green
BA	2.0	+	dark green
BA	4.0	+	dark green
Kinetin	0.5	+	green
Kinetin	1.0	+	green
Kinetin	2.0	+	green
Kinetin	4.0	+	dark green
2iP	0.5	++	dark green
2iP	1.0	++	dark green
2iP	2.0	+	dark green
2iP	4.0	+	dark green
Zea	0.5	-	No response
Zea	1.0	-	No response
Zea	2.0	-	No response
Zea	4.0	-	No response
TDZ	0.5	++++	greyish callusing tissues
TDZ	1.0	++++	reddish and greyish callusing tissues
TDZ	2.0	++++	reddish and greyish callusing tissues
TDZ	4.0	++++	reddish callusing tissues
TDZ	8.0	+++	dark red callusing tissues
TDZ	16.0	++	dark red callusing tissues
TDZ	32.0	++	dark red callusing tissues

**Indicates that are - absent, + poor, ++ fair, +++ good, ++++ very good.*

Auxins such as 2,4-D, NAA, dicamba, picloram (1 mgl^{-1}) were incorporated into the medium supplemented with BAP (4 mgl^{-1}) to study their effect on embryogenic callus production. All cultures were incubated under conditions as described in section 4.2. The growth of callus from leaf explants was estimated by visual observation during 4 weeks in culture. Percentage of the explants responded and mean callus weight of the cytokinin TDZ applied treatments was recorded after 3 weeks in culture.

Out of 10 different auxins tested for callus induction from leaf explants, picloram was found to be the most favourable (data not presented). Picloram at 2 mgl^{-1} produced the highest frequency of response. Dicamba was also as effective as picloram at the tested levels in comparison with other auxins tested. The remaining auxins tested were ineffective for the induction of friable

callus in liquid culture. Poor callus initiation was also observed on media containing the mixture of auxin (2,4-D, NAA, dicamba and picloram) and BAP, and therefore the results were not presented here. However, callus was initiated on most cytokinin supplemented media (Table 56). Of all the cytokinins tested, TDZ was considerably more effective than the rest for stimulating callus initiation and growth with some red pigmentation and deep green in colour. All explants had expanded on all TDZ supplemented media within 7 days after culturing. By day 14, different growth and differentiation responses were obtained on medium supplemented with cytokinins.

Rapidly growing calli were initiated from cultured leaves on all medium formulations containing TDZ and BAP, whereas very little callusing tissues was produced on medium containing K, BA and 2iP. None of the explants produced callus at any concentration of Zea (0.5-4 mgl^{-1}). Callus formed on all leaf explants developed into a mass of friable deep green-yellow with intensive red pigmentation up to 3 weeks of culture on MS medium containing TDZ (Fig. 37). The intensity of callus proliferation was great in medium with 1 mgl^{-1} TDZ alone. The mean callus weight of the explants varied significantly with concentration of TDZ (Table 57). The analysis of the data shows that the average fresh weight of callus was highest at 1 mgl^{-1} TDZ. For fresh weight there was no significant difference for TDZ concentrations between 0.5-4 mgl^{-1}.

Table 57 : Effect of various concentrations of TDZ on induction of calli*.

TDZ mgl^{-1}	Total no of explants inoculated	% of explants* responded	Mean callus weight (g)** ± SE/flask
0.5	20	75	7.12 ± 0.39 a
1	20	80	7.76 ± 0.53 a
2	20	85	6.93 ± 0.59 a
4	20	70	5.98 ± 0.67 a
8	20	60	3.69 ± 0.39 b
16	20	50	1.96 ± 0.26 c
32	20	40	1.76 ± 0.21 c
χ^2 test		P < 0.05	

**After 3 weeks the fresh weight of callus that responded in each flask was recorded. ** The mean values of callus fresh weight is shown ± the sample standard errors of the mean values from two different experiments. Mean values in a column followed by the same letters are not significantly different at p = 0.05 according to the Student t-test.*

However, the use of the cytokinin TDZ with more than 4 mgl^{-1} produced significantly less average fresh weight. Similar mean callus fresh weights were recorded in 16 and 32 mgl^{-1} TDZ. However, high concentrations of TDZ (8, 16 and 32 mgl^{-1}) were generally dark red callusing tissues and the proliferation of callus was little in the culture. There was evidence of a significant difference in the frequencies of explants responding between the TDZ concentrations (Table 57). The proportion of callus induction was 85% in 2 mgl^{-1} TDZ and gradually reduced to 70, 60, 50 and 40% in 4, 8, 16 and 32 mgl^{-1} TDZ, respectively. From this experiment the cytokinin TDZ (in the range of 0.5-4 mgl^{-1}) was then chosen for the induction of callus.

4.2.1.3 : Callus maintenance

The aim of this experiment was to determine the best maintenance medium for callus induced in liquid MS medium supplemented with TDZ (at 0.5-4 mgl^{-1}). To maintain calli, different TDZ treatments were tested by varying the composition of the callus induction media: leaving the calli on the initial medium without any subculture, subculturing the calli on a fresh initial medium, lowering the strength MS medium (1/2), lowering the strength of growth regulators (1/2), subculturing the calli on agar-solidified MS medium with the composition of induction medium, subculturing the calli on a fresh agar-solidified MS medium with different growth regulator (BAP, Kin, 2,4-D and NAA at 1 and 2 mgl^{-1}) from the induction medium (TDZ), supplementing the culture medium with or without 100, 200, 400 and 800 mgl^{-1} banana powder, citric acid and l-ascorbic acid, subculturing the calli on a different culture medium such as WP, SH and G-5, subculturing the calli on a medium supplemented with different carbohydrates such as glucose, fructose, lactose, mannitol and sorbitol. The culture conditions retained for these studies were the same as at the induction stage. The callus friability was recorded after 4 weeks in culture.

Attempts to maintain calli failed when the primary friable callus was transferred to a reduced level of TDZ, a medium with the original concentration of TDZ. Four weeks after the calli were transferred to liquid or agar-solidified MS medium supplemented with the cytokinin TDZ, parts of the calli grew, and newly proliferated fresh callus clumps appeared. Transfer of callus to a liquid MS medium devoid of growth substance was moderately favourable in order to maintain callus. Transfer of callus to an agar-solidified MS medium supplemented with 1 or 2 mgl^{-1} BAP was most favourable in order to maintain calli. During the 4 weeks of subculture the cultures grew more and more rapidly and proliferated new callus clumps with a change of the original colour. Direct transfer of callus from liquid MS medium containing 2 mgl^{-1} to an agarified MS medium having 1 mgl^{-1} TDZ or 1 mgl^{-1} BAP resulted in actively proliferating calli within 4 weeks of culture. Transfer of callus from liquid MS medium to an agar-solidified WP, SH or G-5 media having 1 mgl^{-1} TDZ or 1 mgl^{-1} BAP also resulted in the complete inhibition of callus within 3 days of culture. Transfer of callus from liquid MS medium containing 2 mgl^{-1} TDZ and sucrose to an agarified medium having 1 mgl^{-1} TDZ or 1 mgl^{-1} BAP + fructose, lactose and fructose resulted in the partial inhibition of callus within 3 days of culture, but glucose was as beneficial as sucrose. Transfer of callus from liquid MS medium containing 2 mgl^{-1} TDZ to an agar-solidified MS medium having 1 mgl^{-1} TDZ or 1 mgl^{-1} BAP + mannitol and sorbitol resulted in the complete inhibition of callus within 3 days of culture. The second subculture of callus from agar-solidified MS medium containing 1-2 mgl^{-1} BAP or TDZ to an agar-solidified MS medium having the same growth regulators resulted in globular structures on calli after 4 weeks of culture. Transfer of callus from liquid MS medium containing 2 mgl^{-1} TDZ to an agarified medium having 1 mgl^{-1} TDZ

or 1 mgl^{-1} BAP + 100, 200, 400 and 800 mgl^{-1} banana powder or pineapple powder resulted in the complete inhibition of callus within 3 days of culture. In short, the transfer of callus that was induced in liquid MS medium with TDZ (0.5, 1.0 and 2 mgl^{-1}) from liquid to an agar-solidified MS medium with or without BAP or TDZ (1 or 2 mgl^{-1}) was most favourable in order to maintain callus.

4.2.2 : Regenerated seedling explants: callus production

Nodal bud segments were collected from 1-year-old seedlings of *P. vera* which were growing in a greenhouse. They were surface sterilised as explained in section 3.1.10. Then, they were cultured in agar-solidified MS medium supplemented with 3 mgl^{-1} BAP and 4% sucrose in order to obtain stock material. First pairs of fully expanded leaves from axenic shoot cultures were excised and used as the source of explants. The plant growth media for callus initiation consisted of liquid MS medium, and 0.5, 1, 2 and 4 mgl^{-1} TDZ. All cultures were incubated under conditions as described in section 4.2. Mean callus weight was recorded after 3 weeks in culture.

Table 58 : Effect of various concentrations of TDZ on induction of calli*.

TDZ mgl^{-1}	Total no of explants inoculated	% of explants* responding	Mean callus weight (g)** ± SE/flask
0.5	20	90	6.33 ± 0.37 a
1	20	100	6.67 ± 0.43 a
2	20	100	7.06 ± 0.47 a
4	20	90	6.23 ± 0.51 a

**After 3 weeks the fresh weight of callus that responded in each flask was recorded. **The mean values of callus fresh weight is shown ± the sample standard errors of the mean values from two different experiments. Mean values in a column followed by the same letters are not significantly different at p = 0.05 according to the Student t-test.*

The mean callus weight of the explants did not vary significantly with the concentration of TDZ (Table 58). The analysis of the data shows that the average fresh weight of callus was highest in 2 mgl^{-1} TDZ. For fresh weight there was no significant difference between TDZ concentrations tested. There was also no evidence of a significant difference in the frequencies of explants responding between the TDZ concentrations (Table 58). The proportion of callus induction was 90%, 100%, 100% and 90% in 0.5, 1.0, 2.0 and 4.0 mgl^{-1} TDZ, respectively. When these calli were carefully selected and subcultured on agar solidified MS medium with or without 1 mgl^{-1} BAP at 4 weeks intervals, they retained proliferation capacity for more than a year. The morphological characteristics of calli were the same as the leaf explants derived from axenic germinated seeds.

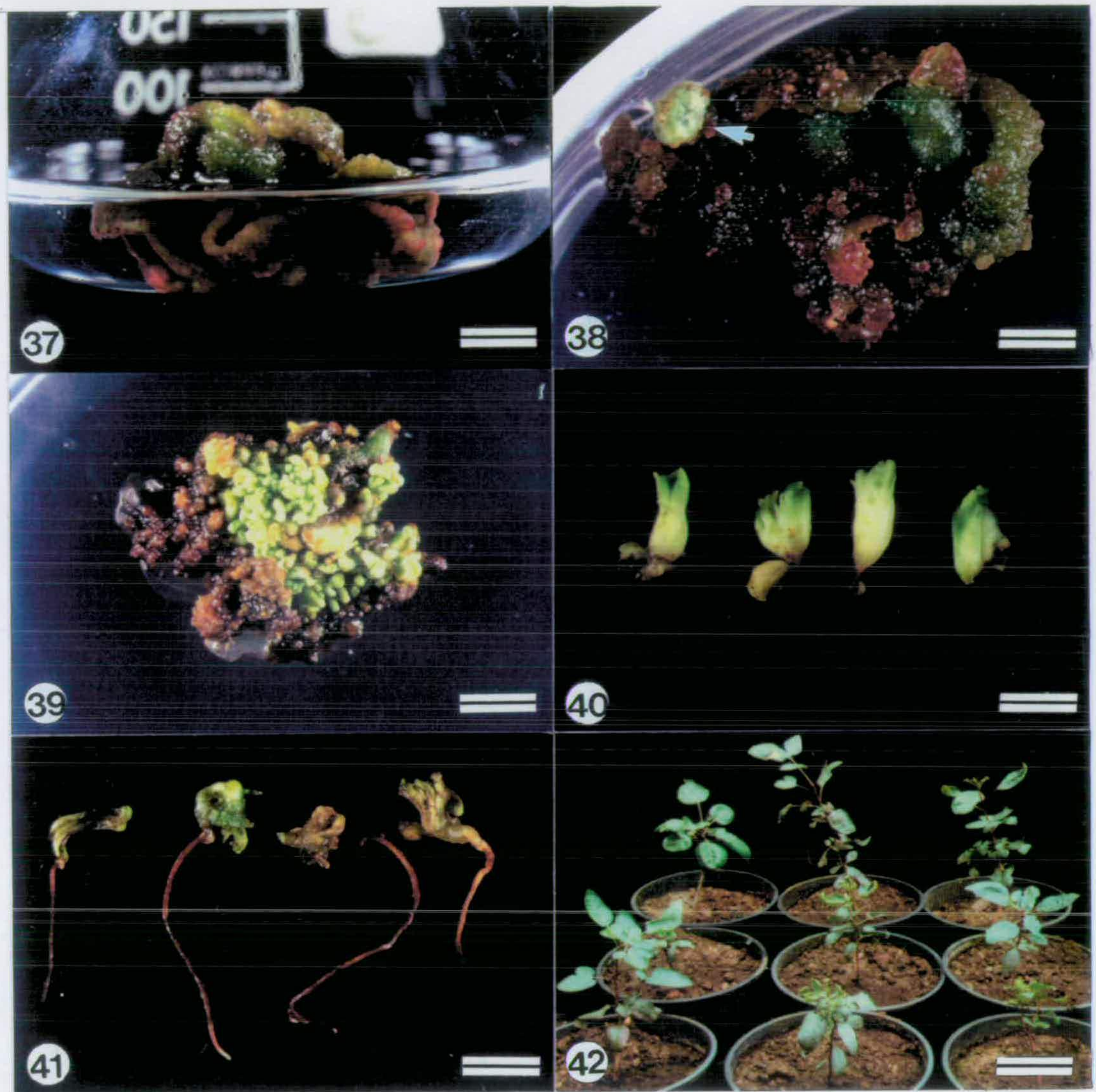
4.2.3 : Mature leaf explants: callus production

Two types of leaf explant were used for callus initiation studies from mature tissues. Mature leaf explants from 50-year-old *P. vera.* were harvested and surface sterilised in 20% NaOCl for 20 minutes, and rinsed three times in distilled water. Leaf explants of 5 mm^2 were cut in and cultured in disposable Petri dishes containing 25 ml of agar-solidified MS medium or, 50 ml liquid MS medium was poured into 250 ml flasks and sealed with a double layer of aluminium foil. The plant growth media for callus initiation consisted of liquid MS medium, and 0.5, 1, 2 and 4 mgl^{-1} BAP or TDZ. Alternatively, mature regenerated-axenic leaf explants from 50-year-old *P. vera* were used as the source of material as explained in section 3.1.10. All cultures were incubated under conditions as described in section 4.2. 20 explants were used to test the effect of each treatment and all experiments were repeated twice.

After 3 or 4 weeks in culture, none of the explants which were harvested from directly mature trees responded to culture in agar-solidified or in liquid MS media. However, the majority of the explants which regenerated via meristem tip culture as explained in section 3.3.1 produced callus or callusing tissues in agar solidified or liquid MS medium supplemented with 0.5-4 mgl^{-1} TDZ 60 day or 30 days after culture, respectively. The callusing tissues in liquid or agar-solidified MS media were generally necrotic because of the release of phenols. The calli produced in these treatments were subcultured on agar-solidified MS medium supplemented with 1 mgl^{-1} BAP for several months, but the development of the embryogenic callus has never been observed.

4.2.4 : Embryogenic cultures

An experiment was initiated to investigate the embryogenic potential of the different leaf explants. The explants (leaves) from axenic germinated seeds (140), regenerated seedlings (80) and mature trees (80) were used for the production of embryogenic calllus. The explants were cultured in 250 ml flasks containing 50 ml of MS medium, 2 mgl^{-1} TDZ, 4% sucrose, 500 mgl^{-1} casein hydrolysate, 50 mgl^{-1} l-ascorbic acid. The cultures were kept for 3 weeks on a gyratory shaker at 98 rmp at 25C under continuous light. The produced calluses were maintained on agar-solidified MS medium supplemented 1 mgl^{-1} BAP. The callusing tissues were regularly subcultured every four weeks. Observations were recorded after 90 days for the number of leaves from which embryogenic callus initiated was determined. In this part of the results, the analysis of the embryogenic callus potential of juvenile (seed and seedling leaf explants) and mature material was given. Embryogenic callus initiation was dependent not only on the age of explants but also on the individual leaf used. There was a significant difference in the frequencies of embryogenic callus obtained between the tested treatments (Table 59, $P < 0.05$).



Figs. 37-42 *: Somatic embryogenesis and plantlet regeneration from juvenile leaf explants of pistachio, P. vera L.:* ***Fig. 37*** *: Induction of greyish-reddish callus in liquid MS medium containing 1 mgl^{-1} TDZ 3 weeks after incubation, bar = 13 mm.* ***Fig. 38*** *: Differentiation of green nodular structures upon transfer of callus from 1-4 mgl^{-1} TDZ to medium containing 1 mgl^{-1} BAP, note the embryogenic centre with yellowish colour (arrow), bar = 5.8 mm.* ***Fig. 39*** *: Polyformation of the embryos on EMS, bar = 10 mm.* ***Fig. 40*** *: Isolated SEs, bar = 3 mm.* ***Fig. 41*** *: Germinated SEs 3 weeks after transfer to germination medium, bar = 7 mm.* ***Fig. 42*** *: Transplanted plantlet, bar = 23 mm.*

Callus that was initiated on any leaf explant did not become embryogenic after subculturing onto agar-solidified MS medium supplemented with BAP or TDZ. Embryogenic calli formed on only 10 leaf explants out of 140 axenic seed originated leaves, 3 leaf explants out of 80 axenic seedling leaves, and none out of 160 leaves from 50-year-old trees. Embryogenic calli were initiated on liquid MS medium containing TDZ (0.5-4 mgl^{-1}) while maintained on agar solidified

MS medium supplemented with or without BAP and TDZ (1 or 2 mgl^{-1}), while agarified hormone-free MS medium was used to mature SEs. Figure 38 shows a differentiation of green nodular structures upon the transfer of calli from 1-4 mgl^{-1} TDZ to medium containing 1 mgl^{-1} BAP. Yellowish nodular structures started to appear after the second subculture onto 1 mgl^{-1} BAP containing medium. TDZ was less effective for the induction of embryogenic competency than BAP despite being more effective for stimulating callus initiation.

Table 59 : Effect of TDZ on induction of embryogenic callus from axenic young leaves from seed, axenic young leaves regenerated from 1-year-old greenhouse grown seedlings and from leaf explants of 50-year-old plant.

Origin of explant	No. of explants cultured	No. of explants with embryogenic callus	% of explants developing embryogenic callus
Leaves from seed	140	10	7
Leaves from seedling	80	3	4
Leaves from mature trees	80	0	0
χ^2 (2 df)			$P < 0.05$

4.2.5 : Embryo development, germination and plantlet growth

After 3 weeks of culture all the calli which developed, were transferred to agar-solidified MS medium supplemented with 1 mgl^{-1} BAP or TDZ and kept on that medium for four weeks, and subcultured regularly every 4 weeks. Observations were recorded for per gram embryogenic callus at the end of 120, 150, 180 and 190 days for SE induction. All cultures were incubated under standard conditions.

At the end of that period (approximately 100 days from the culture beginning) calli showing nodular structures started to produce SEs on MS medium with 1 mgl^{-1} BAP (Fig. 39). SEs were produced in the presence of BAP or TDZ but the response varied in terms of the period of induction and emergence of SEs. SEs were first observed within 4 months originating on the surface of the callus grown in the presence of 1 mgl^{-1} BAP (Table 60). Calli originating from mature material did no show embryoid differentiation. Few SEs were first seen emerging within five months in the presence of TDZ (1 mgl^{-1}) as compared to 4 months with BAP. In fact, the induction of somatic embryogenesis was delayed when 1 mgl^{-1} TDZ was used for embryo development. I reasoned that the SEs may be arrested at the globular stage of development on media that contained TDZ. The frequency of developing embryos in the BAP supplemented medium was higher than the TDZ supplemented medium. Well developed SEs (Fig. 40), were removed from the calli and transferred to a medium containing MS medium without growth regulators with Gamborgh vitamins and sucrose 4% to allow further development. Few SEs

were germinated on a medium containing cytokinins (BAP or TDZ) though most of the SEs formed well-differentiated roots (Fig. 41). The frequency of shoot elongation remained poor in the majority of SEs. When the germinating embryos were cultured on a fresh medium root elongation stopped and at least a couple of tiny leaves formed within 2 weeks.

Table 60 : Number of SEs obtained from leaf culture initiated on TDZ concentrations (1-4 mgl^{-1}) and subsequently transferred to 1 mgl^{-1} BAP containing MS medium and then transferred to MS medium with BAP or TDZ.

	Total no. embryos obtained after							
	120 d		150 d		180 d		190 d	
	BAP	TDZ	BAP	TDZ	BAP	TDZ	BAP	TDZ
Leaves from seed	5	0	13	3	21	8	25	11
Leaves from seedling	3	0	8	5	19	9	19	9
Leaves from mature trees	0	0	0	0	0	0	0	0

**Each value presents the sum of the results of one experiment (After 90 days of culture 1 gram nodular or embryogenic callus per treatment were transferred to MS medium supplemented 1 mgl^{-1} BAP or TDZ and kept on that medium for 100 days).*

Shoot development was increased by using 1 mgl^{-1} BAP, however, at this time the developing emblings started to produce calli from the roots and eventually the whole developing embling recallused. Less than 10% of SEs which were induced in the BAP supplemented media could be regenerated to emblings. However, none of the TDZ supplemented plates did suppress the induction of shoots. In short, in this study it should be noted that BAP and TDZ were found to be most effective for inducing somatic embryogenesis in juvenile leaf explants of pistachio. They were then transplanted to 10 cm diameter plastic pots filled with peat-perlite; the plantlets were maintained at 95% humidity under a glass cover in a growth room for several weeks. Successful acclimatised plantlets were planted in a greenhouse (Fig. 42).

4.2.6 : Discussion

Regeneration of plants *in vitro* was accomplished by somatic embryogenesis only using juvenile-axenic leaf explants. However, the compatibility of the embryo production from EMSes of leaf explants was achieved less frequently than other regeneration materials of pistachio. The effects of conditions of incubation on the production of embryogenic calli were examined. Calli were not formed even at the cut edges of the many explants that were in contact with agar-solidified MS medium in places where the edges of the leaves had curled up and away from the medium during 4 weeks of culture. Even fewer calli were produced 8 weeks after culture. These results for culture initiation are not in agreement with those obtained for *Coffea canephora* by Hatanaka et al. (1991) who initiated embryogenic callus from cultured leaf discs placed on a agar-solidified MS medium. When the leaf explants from the different age groups were cultured under the same conditions, little callusing of tissues was obtained on agar-solidified MS medium but all

leaf explants of pistachio produced callusing tissues in liquid MS medium containing TDZ or BAP after 3 weeks of culture. A similar pattern of callus or EMS initiation was reported for the kernels of immature fruits of pistachio cultured in liquid MS medium supplemented with the cytokinin BAP (Onay et al. 1995a). The effects of plant growth regulators on embryogenesis were examined in leaf cultures of pistachio. Of all the cytokinins tested, TDZ was found to be most effective for callus initiation and growth resulting in some red pigmentation, and deep green in colour. Callus production using the cytokinin TDZ has been reviewed by Huetteman and Preece (1993). In general, the transfer of calli induced in liquid MS medium with TDZ (0.5, 1.0, 2 and 4 mgl^{-1}) from liquid to a agar-solidified MS medium with or without BAP (1 or 2 mgl^{-1}) was most favourable in order to maintain callus. During the 4 weeks of subculture the cultures grew more and more rapidly and proliferated new callus clumps with changes in the original colour. Embryogenic callus initiation was dependent not only on the age of explants but also the individual leaf used, and the type of growth substance and their concentrations. Embryogenic callus formed on 7%, 4% and 0% of axenic seed originated leaves, axenic seedling leaves and axenic regenerated leaves from 50-year-old trees, respectively. The only report of somatic embryogenesis from somatic tissue of a mature gymnosperm tree has involved *C. mexicana* (Chavez et al. 1992). As shown in Table 59, the number of juvenile leaf explants producing embryogenic callus varied significantly in terms of the leaf explants used. Not all calli that were initiated on any leaf explant became embryogenic after subculturing onto agar-solidified MS medium supplemented with growth substances. Embryogenic callus was friable and light to deep green. There was a significant difference in the frequencies of embryogenic callus obtained between the tested treatments (Table 59, $P < 0.05$). TDZ was less effective for the induction of embryogenic competency than BAP despite it being effective for stimulating callus initiation. Cytokinin (BAP) induced embryogenesis in immature fruit cultures of pistachio is reported in the earlier part of this study. In the presence of TDZ, SEs never germinated normally even after prolonged subculture; instead they began to recallus. I reasoned that by producing excessive pigments, it may arrest developing SEs. Perhaps TDZ was inhibiting shoot elongation and SE development although it was the most effective agent for callus initiation. SEs were produced in the presence of TDZ at lower concentrations in the range of 0.5-2 mgl^{-1}. In contrast with my results, regenerated SEs were capable of developing into plants in the presence of TDZ at the concentrations of 1.1-17 mgl^{-1} for *Nicotiana tabacum* (Gill and Saxena 1992).

In these experiments, the number of plantlets (emblings) obtained through embryogenesis remained very low. I attempted to enhance the percentage of plantlet formation through increasing the osmolarility of the medium during experiment studies (unpresented results), since some work has indicated that desiccation may be an important part of the maturation process (Xu et al. 1990) but these experiments did not improve the number of developing embryos. Nevertheless, embryogenic compatibility of leaf explants was not explored in these studies.

Overall, it should be noted that TDZ may be the most potent cytokinin for callus initiation from leaf explants of pistachio and the cytokinin BAP and the age of the explants played a decisive role in the ability to produce embryogenic callus. SEs may be grown into whole plantlets with a low frequency. On the basis of the induction of embryogenesis in pistachio leaf explants by TDZ or BAP alone it may be logically concluded that cytokinin regulated embryogenesis may involve modulation of endogenous cytokinin. However, the question still remains whether mature leaf explants of pistachio have embryogenic potential.

4.3 : CALLUS FRIABILITY AND SOMATIC EMBRYOGENESIS FROM MATURE-STORED SEED EMBRYO CULTURES OF PISTACHIO, *PISTACIA VERA* L.

Mature seeds were used as the source material for callus induction in all experiments. The influence of explant location, sugars, various media and growth regulators on callus friability and somatic embryogenesis was investigated in pistachio. The number of species for which embryogenesis has been achieved in explants from mature trees is still very limited. Thus, in the absence of suitable mature tissues or when older material is non-responsive, the use of zygotic embryos of pollination-controlled plants known to have the desired characteristics may be used for regeneration. The developmental morphology, quantitative aspects of callus differentiation and somatic embryogenesis of zygotic embryos is reported in the following section.

4.3.1 : General culture conditions

Unless otherwise stated, the experimental conditions were as follows:. The explants (seeds) were obtained from mature fruits, harvested in September 1993 from a 30 year-old tree, at Ceylanpinar state production farm, in the Urfa province of South-east Turkey. Mature kernels from which pericarp and shell had been removed were sterilized as explained in section 3.1.7. The testas were removed and washed three times with sterile distilled water. The embryos were extracted and placed in contact with the culture medium. The culture media used for embryonic callus induction consisted of MS medium with Gamborg vitamins, 200 mgl^{-1} casein hydrolysate, 20 mgl^{-1} l-ascorbic acid. The pH of the medium was adjusted to 5.7 with 0.1 N KOH or HCL before autoclaving at 121°C and 120 kPa for 16 min. The medium was solidified with 0.7% agar prior to autoclaving. After 30 days, calli were recultured for 30 days on the same medium composition. One embryo was cultured on 10 ml medium in 50 mm Petri dish, sealed with parafilm. Each treatment was initiated with 10 embryos. Each experiment was repeated at least once. All experiments were incubated at 25°C under a continuous light (25 $\mu mol\ m^{-2}\ s^{-1}$, cool white fluorescent lamps). The final observation is based on surviving explants.

Somatic embryo conversion studies was conducted for the whole of the callus-bearing period. Minitab General Linear Model (GLM) was used to detect significant differences among treatment levels for callus weight per explant. The Student *t*-test was adjusted to separate mean differences when significant treatment effects were detected.

4.3.2 : Effect of location of explants

The influence of embryo orientation to the medium surface was tested to improve the production of embryonic callus. To do so, extracted zygotic embryos were placed onto MS medium containing 2 mgl^{-1} 2,4-D. The percentage of the responding embryos and fresh callus weight per culture was measured after 30 days incubation. Mature zygotic embryos were placed in contact with the culture medium surface in three orientations: a) distal radicle end, b) proximal cotyledonary end, and c) horizontal surface.

Table 61 : Influence of zygotic embryo orientation to the medium surface on embryo response rate and mean callus weights for pistachio after 30 d in culture on MS medium supplemented with 2 mgl^{-1} 2,4-D.

Embryo orientation to medium surface	Embryos responding (%) ± SE*	Mean callus weight per explant (g) ± SE**
Distal radicle end	75 ± 5 a	0.17 ± 0.013 a
Cotyledonary end	65 ± 5 a	0.11 ± 0.019 b
Horizontal surface	95 ± 5 a	0.18 ± 0.032 a

**Each value represents the mean ± SE of the two experiments consisting of 10 explants per treatment in each experiment. **Means within a column followed by a common letter are not significantly different at p = 0.05 according to the Student t-test.*

The proliferation ability was affected by the type of location of explants. The explants incubated in the three orientations produced the callus on media with 4% sucrose but showed a reduction of growth on MS medium with the cotyledonary end-orientated embryos. The calli produced in the 3 orientations were soft and uneven, and white to greyish in colour. Mean scores and general results from each of the three orientations are presented in Table 61. There was no significant difference in the proportion of the embryos responding between the three orientations. The proportion of responding was 95%, 75% and 65% in horizontally surfaced, in distal radicle-end and cotyledonary-end orientated embryos, respectively.

The lowest callus weight and response rate was obtained in cultured embryos that were in contact with the medium with the proximal cotyledonary end which was significantly lower than the distal radicle end and horizontal surface cultured embryos. The weight of callus produced was lower when the distal radicle end and proximal cotyledonary end of the embryos was cultured in contact with the medium. The higher callus weight was obtained in horizontal surface

cultured embryos. The results presented in Table 61 suggest that location of embryos at horizontal surfaces provide suitable conditions for callus initiation of pistachio zygotic embryos *in vitro.*

4.3.3 : Modification of the different sugars supply

Sugars such as sucrose, glucose, fructose and lactose (30 gl^{-1}) were incorporated into the MS medium supplemented with 2,4-D (2 mgl^{-1}), to study their effect on embryogenic callus initiation and embryogenesis.

Embryos cultured in the presence of different carbohydrates and the different sucrose concentration responded within 3 days from the start of incubation. The proliferation ability was affected by sugars and sucrose concentrations. The colour varied depending on the sugar from white to yellowish or greyish. On MS medium with sucrose the callus was more compact and less mucilaginous than on the other sugars tested.

Carbohydrate type significantly influenced callus fresh weight at 30 and 60 days. Table 62 shows a significant difference between the carbohydrate types for callus fresh weight after 30 and 60 days of culture. The largest mean callus weight at 30 days was produced on glucose, which was significantly larger than on fructose and lactose. At 30 days, lactose produced the lowest mean callus weight, which was significantly lower than that of all other carbohydrates.

Table 62 : Influence of carbohydrates on embryo response rates and mean callus weights for pistachio zygotic embryo cultures after 30 and 60-day-treatments in culture supplemented with 2 mgl^{-1} 2,4-D.

Carbohydrates (3% w/v)*	Embryos responding (%) ± SE	Mean callus weight per explant (g) ± SE*	
		30 d	60 d
Sucrose	90 ± 4.08 a	0.225 ± 0.017 ac	0.549 ± 0.041 a
Glucose	87 ± 2.50 a	0.243 ± 0.017 a	0.542 ± 0.040 a
Lactose	85 ± 2.88 a	0.052 ± 0.005 b	0.148 ± 0.015 b
Fructose	90 ± 4.08 a	0.194 ± 0.016 c	0.414 ± 0.038 c

**Each value represents the mean ± SE of the four experiments consisting of 10 explants per treatment in each experiment. **Means within a column followed by the same lowercase letter are not significantly different at p = 0.05 according to the Student t-test.*

There was no significant difference for callus fresh weight between sucrose and fructose. At 60 days, sucrose produced the largest mean callus weight, but not significantly larger than glucose. At 60 days, lactose produced the lowest mean score. Similar responding rates were obtained in cultured embryos that were in MS medium supplemented with different carbohydrates. For a given carbohydrate type there was no significant difference in embryos responding between the

four carbohydrates tested. From these results it may be concluded that lactose has a toxic effect upon the cultured zygotic embryos, which induced lowest callus weight among the carbohydrates tested. Glucose produced the second highest level of mean callus weight but it also displayed higher oxidised polyphenols than sucrose. The data in this experiment have quantified the effects of a range of carbohydrate sources upon the initiation of friable callus. Eventually sucrose was chosen for the further studies.

Morphological changes in the cultured embryos: The first sign of activity (within three days of explanting) was the development of callusing tissues, but within 6 to 10 days of explanting, callusing tissues became visible to the naked eye. Two weeks after explanting, the root tip meristematic regions induced callusing tissues coloured from white to creamy yellow. Three weeks after the initiation of the cultures, friable white to yellowish or greyish callusing tissues were produced from the root tips of the mature embryos. 30 days after explanting, the explants gave rise to several distinct types of callusing tissues depending on the induction medium used. At the end of the 30 th day of the culture, the resulting callusing tissues were transferred to the same medium and growth regulators for the development of embryonic callus. Reculture of the callus onto a medium with the same composition caused rapid growth. 60 days after initiation, depending on the applied treatments, the mature zygotic embryos gave rise to several distinct types of calli on the induction medium: a) uneven and soft, b) sticky and mucilaginous and c) greyish organised and nodular structures developed within the callus which after two subcultures appeared to be entirely composed of embryogenic mass.

At the end of the 60 th day, 50% of cultures from each treatment were transfferred on the initiation medium having the same composition, whereas the remaining cultures were subcultured on MS medium supplemented with the combination of 1-16 mgl^{-1} BAP. A third reculture on the induction medium resulted in unorganised callus whereas callusing in the BAP treatments was more vigorous as compared to any other treatments. In general the callus was surrounded by oxidised phenolic compounds produced in contact with medium of the explants. The composition of the secreted phenolics was not determined.

4.3.4 : Modification of the sucrose supply

To study the effects of a range of sucrose concentrations (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10%) on callus morphogenesis, sucrose was incorporated into the MS medium. The sucrose concentration was maintained throughout the whole culture period, and comparisons were made between the treatment effect on callus morphogenesis and somatic embryogenesis.

In contrast to low concentrations of sucrose, high concentrations of sucrose were associated with large mean callus weights. As in the carbohydrate study, some necrotic callus was observed over time. Noticeably, as sucrose concentration increased, the callus weights also increased. There were slight changes in callus friability associated with sucrose concentrations. The calluses ranged from creamy white to creamy yellow in colour with some red pigmentation. Zygotic embryos cultured in the presence of sucrose at all concentrations tested responded within three days from the start of incubation. Table 63 shows the frequency of embryos responding in cultures of Pistachio zygotic embryos in the callus initiation stage on MS media with different concentration of sucrose. The frequencies of embryos responding did not vary significantly with different concentration of sucrose. The effects of up to 10% sucrose treatment in the culture medium upon callus fresh weight were shown after 30 and 60 days (Table 63).

Table 63 : Influence of sucrose concentration on embryo response (%) and mean callus weights for pistachio zygotic embryo cultures after 30 and 60-day-treatments in culture on MS medium supplemented with 2 mgl^{-1} 2,4-D.

Sucrose (% w/v)	Embryos responding (%) ± SE	Mean callus weight per explant (g) ± SE*	
		30 d	60 d
1	80 ± 10 a	0.10 ± 0.009 a	0.34 ± 0.034 a
2	80 ± 0.0 a	0.11 ± 0.013 ab	0.45 ± 0.056 ab
3	90 ± 10 a	0.11 ± 0.011 ab	0.45 ± 0.045 ab
4	80 ± 10 a	0.14 ± 0.015 bc	0.57 ± 0.069 bc
5	80 ± 0.0 a	0.17 ± 0.022 cd	0.60 ± 0.075 bc
6	80 ± 10 a	0.22 ± 0.027 cd	0.68 ± 0.081 bc
7	95 ± 5.0 a	0.21 ± 0.027 cd	0.70 ± 0.074 c
8	90 ± 10 a	0.24 ± 0.022 cd	0.61 ± 0.065 bc
9	85 ± 5.0 a	0.27 ± 0.047 d	0.62 ± 0.078 bc
10	95 ± 5.0 a	0.19 ± 0.022 cd	0.44 ± 0.038 a

Each value represents the mean ± SE of the four experiments consisting of 10 explants per treatment in each experiment. Different lowercase letters above in a column indicate that these two means are significantly different at p = 0.05 according to the Student t-test.

Sucrose levels significantly influenced callus weight in the tested levels. There were significant differences among tested concentrations for callus weights. The largest mean callus fresh weight at 30 days was produced in the 9 gl^{-1} sucrose treatment, which was significantly not larger than the 5, 6, 7, 8 and 10 gl^{-1} sucrose treatments. The lowest mean callus weights were observed in the 1%, 2% and 3% sucrose concentrations, though significantly lower than callus produced with almost all other applied concentrations. At 60 days, sucrose at 7 gl^{-1} produced significantly larger mean callus fresh weight that the 1, 2, 3 and 10 gl^{-1} sucrose treatments. These results suggest that sucrose concentrations from 5% to 9% may be adequate for callus initiation. In addition, increasing the concentration of sucrose in the culture up to 10% did not

increase oxidised polyphenols during incubation. Higher concentrations of sucrose resulted in larger mean callus weights at both 30 and 60 days. Therefore, 4% of sucrose may be used as a minimal level for callus initiation.

4.3.5 : Modification of different media supply

This experiment was designed to assess the effect of different media on callus initiation and somatic embryogenesis. To study the effect of different media on somatic embryogenesis the basal media (MS, SH, WP and G-5) were supplemented with 2 mgl^{-1} 2,4-D and 4% sucrose.

There was evidence of a significant difference in the frequencies of embryos responding between the results obtained from the different media (Table 64). Embryos cultured in the presence of MS, SH, WP and G-5, in all the media tested, responded within three days from the start of incubation. The MS and G-5 media gave the best results, with higher responding level of 95% while the lowest proportion of responding embryos with SH (82.5%) medium.

Table 64 : Influence of media on embryo response rates and mean callus weights for pistachio zygotic embryo cultures after 30 and 60-day-treatments in culture supplemented with 2 mgl^{-1} 2,4-D.

Media	Embryos responding (%) ± SE	Callus weight per explant (g) ± SE*	
		30 d	60 d
MS	95.0 ± 2.88 a	0.22 ± 0.015 a	0.475 ± 0.027 a
SH	82.5 ± 2.50 b	0.17 ± 0.015 b	0.366 ± 0.028 b
WP	92.5 ± 4.78 ab	0.14 ± 0.013 b	0.360 ± 0.028 b
G-5	95.0 ± 2.88 a	0.13 ± 0.016 b	0.274 ± 0.023 c

**Each value represents the mean ± SE of the four experiments consisting of 10 explants per treatment in each experiment. Different lowercase letters above in a column indicate that these two means are significantly different at p = 0.05 according to the Student t-test.*

Media formula significantly influenced callus weight at each subculture (Table 64). The largest mean callus weight at 30 days was produced on MS medium, which was significantly larger than all other media tested. The response on SH medium was significantly lower than on all other tested media. At 60 days, MS medium produced again significantly larger mean callus weight than the rest of the media tested. There was no significant difference in the mean callus weight between SH and WP. The lowest mean callus weights occurred in B-5, which was significantly lower than all other media tested.

4.3.6 : Modification of growth regulators supply

To study the effect of various auxins and cytokinins on embryogenesis, auxins such as 2,4-D, NAA, 2,4,5-T, IBA (0, 1, 2, 4 mgl^{-1}) and cytokinins such as BAP and TDZ were modified in the culture medium during callogenesis. I also compared the effects of a range of 2,4-D concentrations (1, 2, 4 and 8 mgl^{-1}) with BAP concentration remaining constant at 2 mgl^{-1} and, conversely, of a range of BAP concentrations (1, 2, 4 and 8 mgl^{-1}) with 2 mgl^{-1} 2,4-D and 4% sucrose.

4.3.6.1 : Cytokinins

The cytokinins tested were not capable of initiating callus from zygotic embryos. The explants cultured in the cytokinin applied treatments did not convert the intact explants into callusing tissues during 30 days of culture. Therefore, the cytokinins were not used in the induction of embryogenic callus. When the explants were kept on the same medium for more than 30 days some explants produced callus in some treatments tested. However, the cytokinin BAP was the most favourable growth regulator to maintain calli.

4.3.6.2 : Auxins

Auxin concentrations alone had a significant effect on callus weights on MS medium (Table 65). Among the auxins tested, NAA and IBA were not capable of inducing callus from zygotic embryos. In those treatments, all zygotic embryos produced shoots with a piece of callus at the root tips.

Table 65 : Mean callus weights for pistachio zygotic embryo cultures after 30 and 60-day-treatments of auxins*.

Auxin	Concentration (mgl^{-1})	Mean callus weight per explant (g) ± SE** 30 d	60 d
2,4-D	1	0.14 ± 0.018 a	0.24 ± 0.038 a
2,4-D	2	0.15 ± 0.018 a	0.37 ± 0.048 b
2,4-D	4	0.14 ± 0.018 a	0.32 ± 0.036 ab
2,4,5-T	1	0.26 ± 0.030 b	0.52 ± 0.051 a
2,4,5-T	2	0.15 ± 0.028 a	0.30 ± 0.045 b
2,4,5-T	4	0.14 ± 0.017 a	0.27 ± 0.039 b

**Data on IBA and NAA have not been represented because these auxins did not convert all the explants into callus. **Each value represents the mean ± SE of the two experiments consisting of 10 explants per treatment in each experiment. Different lowercase letters in a column indicate that these two means are significantly different at p = 0.05 according to the Student t-test.*

Out of the four auxins (2,4-D, NAA, 2,4,5-T and IBA) tested for embryonic callus initiation from zygotic embryos, 2,4-D and 2,4,5-T were found to be the most favourable, 2, 4,5-T at 1 mgl^{-1} at 30 and 60 days produced the largest callus weight, which was significantly larger that obtained from all the other treatments tested. At 30 day, there was no significant difference between mean callus fresh weight per explant for the different concentrations of 2,4-D. However, there was a significant difference between mean callus fresh weight per explant for the different concentrations of 2,4,5-T. The 1 mgl^{-1} 2,4,5-T treatment produced higher callus weight, which was significantly larger than the rest of the 2,4,5-T concentrations tested. At 60 day, there was a significant difference between mean callus fresh weight per explant for the concentrations of 2,4-D or 2,4,5-T. The 1 mgl^{-1} 2,4,5-T produced the largest callus weight which was significantly larger than that obtained from the rest of the treatments tested. The 2 mgl^{-1} 2,4-D produced the largest callus which was significantly larger than that obtained in the 1 mgl^{-1} 2,4-D.

Table 66 : Mean callus weights for pistachio zygotic embryo cultures after 30 and 60-day-treatments of 2-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BAP).

2,4-D:BAP combinations (mgl^{-1})	Responding embryos (%)	Callus weight per explant (g)± SE* 30 d	60 d
1:2	85 ± 5.0 a	0.10 ± 0.019 a	0.24 ± 0.051 ab
2:2	100 ± 0.0 a	0.18 ± 0.029 b	0.40 ± 0.058 c
4:2	85 ± 5.0 a	0.07 ± 0.013 a	0.17 ± 0.031 a
8:2	85 ± 15 a	0.07 ± 0.017 a	0.20 ± 0.039 ab
2:1	95 ± 5.0 a	0.19 ± .031 b	0.38 ± 0.062 bc
2:4	100 ± 0.0 a	0.16 ± 0.027 b	0.27 ± 0.029 bc
2:8	90 ± 10 a	0.07 ± 0.010 a	0.14 ± 0.017 a

**Each value represents the mean ± SE of the two experiments consisting of 10 explants per treatment in each experiment. Different lowercase letters above in a column indicate that these two means are significantly different at p = 0.05 according to the Student t-test.*

The effect of combination of 2,4-D and BAP on mean callus weight is presented in Table 66 Plant growth regulator combinations significantly influenced callus weights at each subculture. At 30 days, the combination of 2 mgl^{-1} 2,4-D and 1 mgl^{-1} BAP produced the largest callus weight, though not significantly different from the combination of 2 mgl^{-1} 2,4-D and 1, 4 mgl^{-1} BAP. Higher levels of 2,4-D or BAP 8 mgl^{-1}, in combination with BAP or 2,4-D, decreased the mean callus weight (Table 66). Mean callus weights were similar for the combination of 2 mgl^{-1} 2,4-D and 1, and 2 mgl^{-1} BAP at the second subculture. In general, callus weights increased more than double befor day 60.

4.3.7 : Maintenance of calli

To determine the optimum medium for callus maintenance, after two recultures at 30 day intervals, half of the calli derived from all of the experiments were subcultured onto the same initiation medium or transferred to MS medium supplemented with BAP (1, 2, 4, 8 and 16 mgl^{-1}), 4% sucrose and 0.7% agar.

In all the treatments tested, there was maintenance of non-friable calli beyond day 60 in culture when the calli were subcultured on the same medium, but only the use of MS medium supplemented by various BAP concentrations gave maintenance of friable calli. In general when calli were subcultured onto the same or different media, they stopped growing and turned brown. Thus, non-friable calli show early browning and can be readily identified and removed prior to the time of transfer to a secondary medium.

In the BAP treatments, calli grew further. The different auxins and cytokinins during proliferation affected the maintenance of callus. The calli cultured in the BAP supplemented medium spontaneously proliferated greyish calli, which were compact and less mucilaginous than any other tested treatments. The best condition for callus maintenance was on the MS medium supplemented with 4% sucrose and BAP (1, 2 and 4 mgl^{-1}). BAP alone at the different concentrations tested maintained and proliferated hard green callus with red pigmentation. These calli were subcultured and maintained for more than one year on the same medium. The higher concentrations of BAP in the MS medium proved the most effective means of inducing red pigmentation. When the BAP concentration was increased the induced calli had red pigmentation and showed no browning.

4.3.8 : Development of somatic embryos

To stimulate somatic embryo development, friable calli grown for 30 days on the maintenance treatments were transferred to the same maintenance medium or different concentrations of BAP (at 1, 2 and 4 mgl^{-1}). This study was carried out throughout the calli-bearing period.

Two months after initiation, of the treatments tested half of the calli were recultured to BAP (at 1, 2, 4, 8 and 16 mgl^{-1}). The calli exhibited two distinct characteristics: change in proliferation ability and colour. The colour varied from white to yellowish and green to reddish or to deep green depending on the growth regulator used for initiation. The 1, 2 and 4 mgl^{-1} BAP treatments spontaneously formed deep green or greyish callus.

Somatic embryos were never observed when the explants were incubated continuously in the original callus induction media supplemented with cytokinin or auxin. Expression of somatic embryos was only observed when the explant was initiated in the MS medium supplemented with 1-4 mgl^{-1} 2,4-D or its combination with BAP, recultured having the same composition and maintained on a medium, which contains 1-2 mgl^{-1} BAP. Nodular structures developed within the callus after 2 subcultures. Occasionally, these green nodules differentiated into clusters of somatic embryos. The production of somatic embryos with BAP and its combination with 2,4-D may be due to a favourable interaction between BAP and 2,4-D in the initiation stage. After a few subcultures, various developmental stages of somatic embryos could be observed from the early stages of embryos to the formation of well developed embryos at the external part of the callus. Mature somatic embryos are bipolar with a radicle and cotyledons and are thus to the zygotic counterpart. As the somatic embryos developed further, some took on the appearance of normal embryos but most of them were swollen. Within the embryogenic calluses obtained, there was no formation of somatic embryos beyond months 6 in culture. Germination of somatic embryos was carried out on MS medium devoid of growth regulators and under continuous light. When the somatic embryos were detached from the callus and placed on a growth regulator-free medium they were able to develop into normal-looking plantlets. Plantlets with elongated shoots were transferred to soil and cultured in the greenhouse. Few plantlets produced *in vitro* survived transplanting after 3 months of transplanting.

4.3.9 : Effects of genotypes on somatic embryogenesis of *P. vera* L.

In the first part of the study, the influence of explant location, sugars, various media and growth regulators on callus friability and somatic embryogenesis were investigated in *Pistacia vera* L. A concentration of 1-4 mgl^{-1} 2,4-D was found to be a most satisfactory growth regulator for producing viable callus and this may be used routinely in callus induction from zygotic embryos, but the cytokinin BAP was the most effective growth regulator to maintain callus. In the second part of the study, eight of the main Turkish pistachio cultivars (Antep, Siirt, Bilgin, Kellegouchi, Mumtaz, Ohadi, Sedifi, and Vahidi) were tested for the induction of somatic embryogenesis. A 2 mgl^{-1} 2,4-D was used for callus induction. After 2 recultures on the same medium, the effect of BAP, and 2,4-D at 1 and 2 mgl^{-1} was tested for capacity to induce embryogenic callus. Callus initiation studies were conducted over a 30-day period. At the end of the 30th day, the % of the mature zygotic embryos responding was recorded for each genotype. Calli were aseptically weighed after 30 and 60 days. Callus morphology was assessed weekly up to 90 days, and calli bearing clearly visible proembryos were scored. The percentage of zygotic embryos bearing embryogenic callus (EC) and number of mature SEs per gram EC were recorded.

Figure 43 shows excised mature zygotic embryos of genotype Antep before placement on the culture medium. The first sign of activity (within three days of explanting) was the development of callusing tissues, but within 10 days of explanting, callusing tissues became visible to the naked eye (Fig. 44). Two weeks after explanting, the root tip meristematic regions induced callusing tissues which were coloured from white to creamy yellow. Three weeks after the initiation of the cultures, friable white to yellowish or greyish callusing tissues were produced from the root tips of the mature embryos. 30 days after explanting, the explants gave rise to different types of callusing tissues depending on the induction medium used. At the end of the 30 th day of the culture, the resulting callusing tissues were transferred to the same medium with growth regulators for the further development of callusing tissues. Reculture of the callus onto a fresh medium with the same composition induced rapid growth. 60 days after initiation, depending on the applied treatments, the mature zygotic embryos gave rise to several types of calluses on the induction medium: white, uneven and yellowish organised or disorganised.

At the end of the 60 th day, 50% of the cultures from each treatment were subcultured on an initiation medium having the same composition (MS medium supplemented with 2 mgl^{-1} 2,4-D), whereas the remaining cultures were subcultured on MS medium supplemented with 1 or 2 mgl^{-1} BAP or 2,4-D. A third reculture on the induction medium resulted in unorganised callus whereas callusing in the BAP treatments was more vigorous as compared to the 2,4-D treatments. In general, the callus produced was surrounded by a ring of oxidised phenolic compounds secreted into the medium. The calluses cultured in the BAP-supplemented medium spontaneously proliferated greyish calluses. The calluses exhibited two distinct characteristics: change in proliferation ability, and change in colour. The colour varied from white to yellowish and green to reddish or to deep green depending on the concentrations of BAP used for maintenance. Nodular structures were developed within the callus of the three genotypes (Antep, Siirt and Sedifi) which, after two subcultures on MS medium containing 1 or 2 mgl^{-1} BAP, appeared to be entirely composed of embryogenic calluses. Table 67 shows the % of zygotic embryos responding in cultures of pistachio genotypes in the callus initiation stage on MS medium supplemented 2 mgl^{-1} 2,4-D 30 days after culture. The % of embryos responding did not vary significantly within different pistachio genotypes.

The effects of pistachio genotypes in the culture medium upon callus fresh weight were also shown after 30 and 60 days (Table 67). Pistachio genotypes significantly influenced callus fresh weights at each subculture. At 30 days, only the genotype Ohadi produced significantly lower callus weight, though not significantly different from genotype Kellegouchi. The largest mean callus weight at 60 days was produced on the genotype Sedifi, which was significantly larger than all other genotypes tested, but there was no significant difference in the mean callus weight between Vahidi and Sedifi.

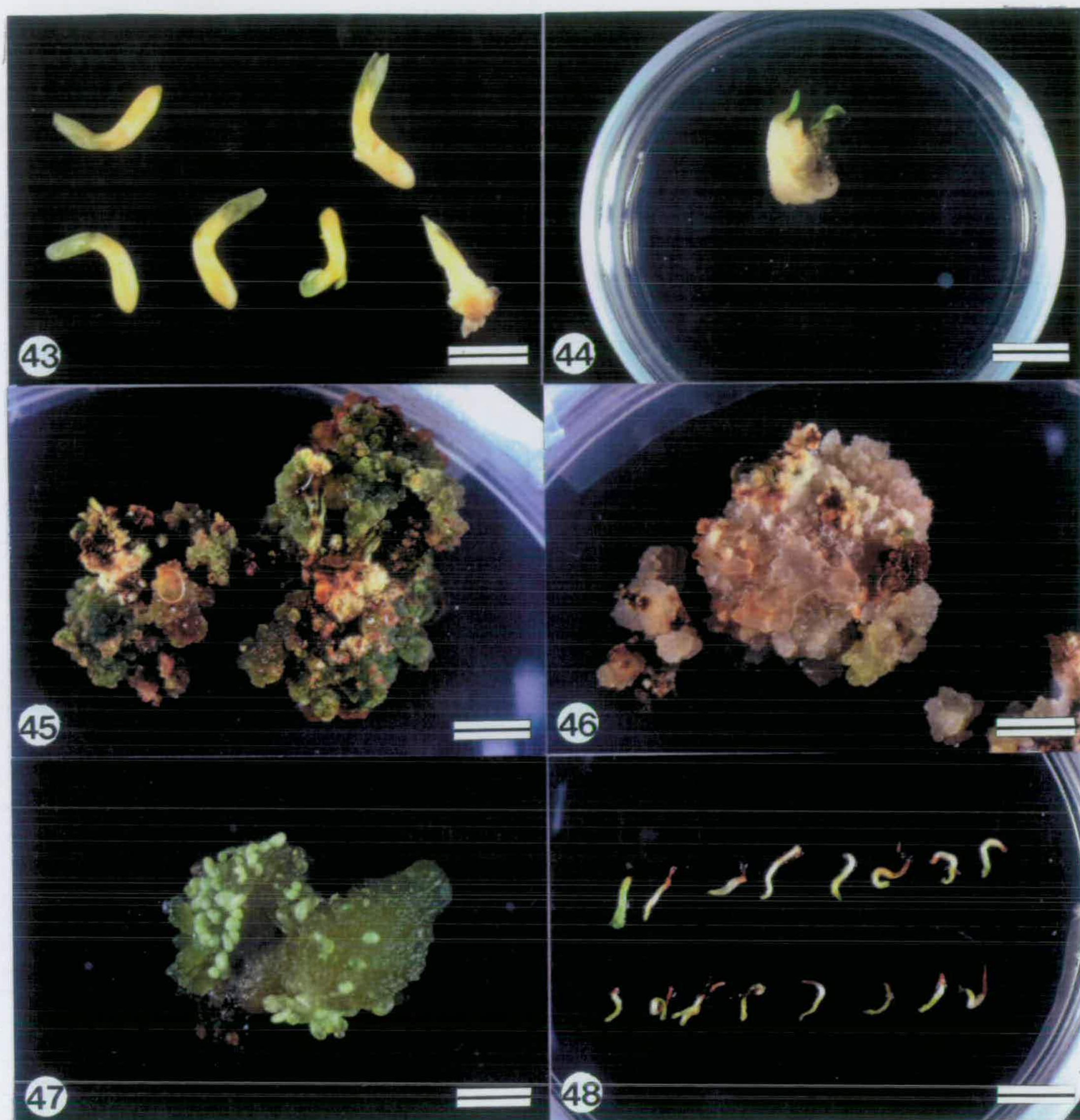
Table 67 : Effect of genotype on embryos responding (%), callus fresh weight, percentage of embryos with embryogenic callus and number of mature SEs per 1g embryogenic callus.

Genotype[1]	ER (%) ± SE[2]	CFW per explant (g) ± SE[3]		% of embryos with EC[4]	No. of mature SEs[5]
		30 d	60 d		
Antep	90 ± 3.0	0.14 ± 0.012 b	0.48 ±0.022 b	10	17
Siirt	90 ± 10	0.16 ± 0.013 b	0.60 ± 0.058 bc	15	15
Bilgen	83 ± 3.5	0.19 ± 0.014 b	0.57 ± 0.061 bc	0.0	0.0
Kellegouchi	86 ± 6.5	0.12 ± 0.034 ba	0.65 ± 0.075 c	0.0	0.0
Ohadi	77 ± 3.5	0.10 ± 0.012 a	0.34 ± 0.024 a	0.0	0.0
Vahidi	83 ± 10	0.17 ± 0.013 b	0.72 ± 0.074 cd	0.0	0.0
Sedifi	90 ± 10	0.19 ± 0.009 b	0.84 ± 0.071 d	16	24
Mumtaz	87 ± 0.0	0.18 ± 0.018 b	0.57 ± 0.034 c	0.0	0.0
χ^2 (7 df)				P < 0.05	

CFW: Callus fresh weight, ER: Embryos responding, EC: Embryogenic callus, SEs: Somatic embryos.
[1]*Each treatment was initiated with 15 embryos with two repetitions.*
[2]*The data were recorded after 30 days in culture at 25°C.*
[3]*Treatment means with the same letters are not significantly different at p = 0.05.*
[4]*The data were collected after 4 months initiation.*
[5]*The data were recorded from one experiment after 30 days on maturation medium.*

At 60 days, Ohadi again produced significantly lower mean callus weight than the rest of the genotypes tested. Embryogenic callus initiation was dependent not only on the genotype and growth regulator, but also varied among the individual zygotic embryos within genotypes. It was established that each of the examined genotypes was not able to produce embryogenic tissue. The induction frequency of embryos with EC was in the range of 10-16% in responding genotypes (Table 67). There were significant differences in the frequencies of embryogenic callus obtained between the genotypes tested (Table 67, P < 0.05).

From these results it may be concluded that different genotypes may require different culture conditions to fulfil somatic embryogenesis, and that optimal culture conditions should be defined for the best performance of each genotype. Embryogenic callus formed on only the Antep, Siirt and Sedifi genotypes. Embryogenic calluses were never observed when the explants were incubated in the original callus induction media supplemented with auxin (2,4-D). Callus that was initiated on the majority of explants (embryos of different genotypes) did not also become embryogenic after subculturing onto agar-solidified MS medium supplemented with BAP. Agar-solidified MS medium containing 1 or 2 mgl^{-1} BAP was only medium on which embryogenic calli could be initiated. They were maintained on agar solidified MS medium supplemented either 1 or 2 mgl^{-1} BAP and agarified growth regulator-free MS medium was used for the maturion and elongation of single embryos. As reported in the first part of the study, none of the other tested auxins and cytokinins were able to produced embryogenic callus. Nodular structures developed within the callus after 2 subcultures. The green embryogenic callus (Fig. 45) was easy to distinguish from yellowish non-embryogenic callus (Fig. 46).



Figs. 43-46 : Somatic embryogenesis in cultured zygotic embryos of a Turkish commercial cultivar Antep (P. vera L.) :Fig. 43 : *Excised mature zygotic embryos of pistachio, P. vera L. Bar = 5 mm.* ***Fig. 44*** *: Callusing tissues became visible to the naked eye after 7 days of culture. Bar = 8.5 mm.* ***Fig. 45 :*** *Embryogenic callus of P. vera initiated from zygotic embryo on modified MS medium containing 1 mgl^{-1} BAP. Bar = 6.6 mm.* ***Fig. 46 :*** *Non-embryogenic callus of P. vera initiated from zygotic embryo on modified MS medium containing 2 mgl^{-1} 2,4-D. Bar = 6.6 mm.* ***Fig. 47 :*** *The early stages of somatic embryos to the formation of well developed embryos at the external part of the callus on MS medium. Bar = 3.5 mm.* ***Fig. 48 :*** *Germinated somatic embryos on MS medium devoid of growth regulator. Bar = 10 mm.*

Occasionally, these green nodules differentiated into clusters of somatic embryos. After a few subcultures, various developmental stages of somatic embryos could be observed, from the early stages of embryos to the formation of well developed embryos at the external part of the callus (Fig. 47). Mature somatic embryos are bipolar with a radicle and cotyledons. As the somatic embryos developed further, some took on the appearance of normal embryos but most of them were swollen. The proliferation rate was low so that it was easy to identify the original somatic embryos. Within the obtained embryogenic callus there was no formation of somatic embryos beyond month 6 in culture. Germination of somatic embryos (Fig. 48) was carried out on MS medium devoid of growth regulator and under continuous light. When the somatic

embryos were detached from the callus and placed on a growth regulator-free medium they were able to develop into normal-looking plantlets. Within 6 weeks, about 10% of the embryos cultured in the light had developed into plantlets while the remaining somatic embryos became swollen or recallused. Plantlets with multiple shoots were occasionally observed.

4.3.10 : Discussion

These studies have shown that callus differentiation from zygotic embryos was dependent on explant location, hormonal conditions, media, sugars, and their concentrations in the medium. Although auxin alone was sufficient in inducing callus, BAP at a low concentration (1-2 mgl^{-1}) was found to be helpful in improving the callus growth. The best conditions for callus initiation from the extracted zygotic embryos of pistachio in this study were on MS medium with relatively high sucrose concentrations (5-9%) and 2 mgl^{-1} 2,4-D or in combination with 2 mgl^{-1} BAP (Table 66). Among the different carbon sources tested for induction of embryogenic callus in pistachio, sucrose evoked the best growth response followed by glucose and fructose, while lactose was completely ineffective. The most commonly used carbohydrate for plant tissue culture is sucrose. Even sucrose was not always beneficial for somatic embryogenesis in the present study, although in peanut (Eapen and George 1993), it induced the most favourable conditions for somatic embryogenesis. The best medium tested in our study (MS) contained high levels of nitrogen compounds and Gamborg vitamins while SH, WP and B-5 medium did not. However, attempts to establish suspensions of embryogenic cell clusters failed. Although 2,4-D is considered the most potent auxin for the induction of embryogenic callus and formation of somatic embryos (Ammirato 1984), here no somatic embryo formation occurred on MS medium containing only 2,4-D. In a woody species *Euonymus europaeus* L., in the presence of 2,4-D, the expression of somatic embryogenesis was never observed and the sensitivity to an exogenous signal and the ability of explants to form somatic embryos were associated with the conservation time of seeds (Boneau et al. 1994). In the present study BAP was found suitable for the expression of somatic embryogenesis in *P. vera* L. Studies on different explants of pistachio indicated that the various types of explants used may have different requirements for callus induction but BAP was essential for the expression of somatic embryogenesis. However, the concentrations of BAP in the culture medium were critical and lower concentrations of BAP (1-4 mgl^{-1}) in the culture medium were necessary for both callus maintenance and embryoid initiation from zygotic embryos. Embryogenic callus was never observed when the callus was subcultured on the original induction media. The formation of embryoids directly on the zygotic embryo derived callus occured at quite a low incidence. Therefore, from these cultures further studies were not conducted.

As previously reported in section 4.1, EMSes were obtained from immature seed explants of pistachio using the cytokinin BAP. In section 4.2 I have also demonstrated that TDZ was the most effective agent for the induction of callus from leaf explants of pistachio but BAP was again essential for the expression of somatic embryogenesis. Once an embryogenic tissue is obtained, development or maturation of somatic embryos may also be carried out in the presence of a cytokinin or auxin in the culture medium. Plant growth regulators may not be necessary for embryo development and germination. These results show that the cytokinin BAP is essential for the induction of embryogenic callus from mature zygotic embryos of *P. vera* L. This study has also shown that there is zygotic embryo to zygotic embryo variation for the initiation of embryogenic callus within the genotype. With mature embryo explants, the genotype often has a distinct effect on embryogenesis (Bonga and Aderkas 1992). The variation observed in potential for embryogenic tissue formation implies that optimal culture conditions should be defined for the best performance of each genotype. The ability of different genotypes to produce embryogenic tissue has previously been demonstrated in some conifer species (Jain et al. 1989, Cheliak and Klimaszewska 1991). As the developing somatic embryos were never observed beyond month 6, when the explants were incubated in the presence of a cytokinin or auxin, the presented results suggest that although somatic embryogenesis in pistachio can occur in several pathways using different explants and growth regulators, some schemes are not very efficient. Though the observation of the chromosomes demonstrated that regenerated plantlets did not show any chromosomal abnormality (see Chapter 6), the regenerated plants from zygotic originated tissues were of unproved genotypes. Therefore this does not allow for the use of somatic embryogenesis in a cloning strategy but a model system for the preliminary genetic improvement programmes for pistachio.

CHAPTER FIVE
CYTOLOGICAL STUDIES

CHAPTER FIVE : CYTOLOGICAL STUDIES

The first research on the chromosome number of *P. vera* L. (2n = 30) was reported by Zohary (1962). Since then, other researchers have also reported the same chromosome numbers for *P. vera* L. (Maggs 1973, Ozbek 1963). However, the chromosome of *P. vera* L. have been reported as 2n = 32 (Barghchi and Alderson 1989). There does not appear to have been any work on chromosome morphology or sex determination. The chromosomes of plant cells cultured *in vitro* are generally subject to important numerical and structural variations. The analysis of these chromosome variations can be envisaged only if sufficient information is acquired concerning the karyotypes of the species involved in the propagation.

The objectives of the present study were to (i) provide information on analytical techniques in plant cytogenetics; (ii) characterise more completely the chromosomal complement of *P. vera* by using meristematic root tips for improved mitotic preparations; (iii) define the karyotype and idiogram of *P. vera* L. according to the recommended cytogenetic nomenclature and standards; (iv) and then apply the developed techniques to somatic embryos of pistachio to provide information on the chromosome structure of somatic embryos. Reagents used for chromosome preparations are presented on Table 68.

5.1 : Plant material and sampling

In the present study zygotic and somatic embryos were used. The plant materials studied are presented in Table 69. Seeds of pistachio were surface sterilised as explained in section 3.1.7. and germinated *in vitro* conditions as explained in section 4.1.6. Cuttings of 50-year-old male trees rooted neither *in vitro* nor *in vivo*. Somatic embryos were produced from EMS derived from immature fruits, EMS derived from juvenile leaf explants and embryogenic callus derived from zygotic embryo explants of pistachio, *Pistacia vera L.* Hundreds of somatic or zygotic embryos were germinated aseptically in Petri dishes on the MS medium, maintained under the same conditions. To make later measurements possible, different roots were sampled as follows: a) first root tip up to 2 mm long; b) second root up to 2mm long and at different times of day. The number of cells observed in division was variable but in general quite low. Therefore, to find the best conditions of germination and times of sampling the materials in order to get the most active meristems, the different combinations of the above conditions were tested.

It seems there is no difference in the number of divisions observed in roots sampled at the different times, and between main and axillary roots. To obtain clearer slides, the sampling material must be less than 1 mm thick because the greater thickness of the root tissues makes it more difficult to avoid cell overlapping.

Table 68 : List of the reagents and their method of preparation used in chromosome studies with reference to proprietary sources of chemicals in brackets where helpful.

1)	1-Bromonaphthalene (BDH). Saturate aqueous solution.
2)	8-Hydroxyquinolene (Sigma). 0.002 M aqueous solution.
3)	Colchicine (Sigma): 0.1-0.5%
4)	p-Dichlorobenzene (Sigma). Saturated aqueous solution.
5)	Farmer's fixative. 3 (ethanol) : 1 (acetic acid).
6)	Carnoy's fixative. 6(ethanol) : 3 (acetic acid) : 1 (chloroform). Must be prepared fresh.
7)	Lacto-propionic orcein. Stock solution: 2g natural orcein (Sigma) dissolved overnight in a mixture of 50 ml lactic acid and 50 ml propionic acid. Filter. Working solution: dilute stock solution to 45% with water.
8)	Acid acetic orcein. 1 (HCL) : 9 (Acetic orcein). Must be prepared fresh.
9)	Feulgen's stain: A. Add 0.7 g basic fuchsin (BDH/GURR 34088) and 3.8g sodium metabisulphate to 200ml of 0.15NHCL. B. Place mixture on a magnetic stirrer for 2-3 hours at room temperature. At the end of this period, the solution should be straw-coloured. C. Decolourize with 1g activated charcoal by shaking. D. filter and make up to 200ml with distilled water. The solution should be as clear as water. E. The pH of the solution should be about 2.2. F. Store in the fridge in a tightly stoppered bottle.
10)	Cellulase (Sigma). Prepare a 4% solution with a piece of Thymol to avoid fungal growth. Can be used for 1 week.
11)	Pectinase (Sigma). Prepare a 4% solution with a piece of Thymol to avoid fungal growth. Can be used for 1 week.

Table 69 : Material examined or cytology by the present author, its origin and part of plant studied.

Species	Country	Part of plant studied
Cultivar 'Antep' of *Pistacia vera L.* (Zygotic embryos of female plants)	Turkey	Root tip
Somatic embryos produced from EMS derived from immature fruit *Pistacia vera L.*	Turkey	Root tip
Somatic embryos produced from EMS derived from juvenile leaf explants of *Pistacia vera L.*	Turkey	Root tip
Somatic embryos produced embryogenic callus derived from zygotic embryo explants of *Pistacia vera L.*	Turkey	Root tip

5.2 : Cytological treatments

Root tips were taken from young plants after germination of zygotic embryos or young embling after germination of somatic embryos. The root tips were then prefixed with one of the following solutions: 0.1-0.5% colchicine (1-6 h); 0.1-1% α-bromonaphthalene (1-10 h); 0.002 M hydroxyquinoline (1-6 h). The prefixation was done at room temperature. The prefixed root tips were rinsed in running water and immersed in a fixative solution of alcohol acetic (3 vol : 1 vol),

for analysis using the squash method. The root tips were rinsed in distilled water after 24 h fixation. They were then hydrolysed for 10-60 min in 5 N HCl at room temperature, following which they were stained with lacto-propionic-orcein and squashed.

Table 70 : Technique for preparing cytological slides for chromosome counting in root tips.

Technique stage	Steps	Time temp-erature
Pre-treatment	1.1-bromo-naphthalene	4 hours at room temp.
Fixation	2. Farmer's fluid	24h at room temp.
Storage	2.a. 70% ethanol	a few days at room temp.
Hydrolysis/ Maceration	3. 5N HCl 4. Wash in dis-tilled water 6. Cellulase 4% pectinase 4% (1:1) 7. Transfer the root to a slide	30 min at room temperature a few min. few minutes
Staining	8. lacto-propionic orcein 9. Cut the densely stained root tip (top millimetre of the root and place it in a drop of lactopropionic orcein) 10. Tap or tease 1 mm piece of tissue in 5 mm drop of lacto-propionic orcein. Remove visible particles.	5 min
Squashing	11. Cover with a no. 0 cover-glass 12. Turn the slide up side down and press it against 3 sheets of filter paper 13. Turn the slide up again and tap the coverglass with blunt metal rod 14. heat the slide gently	

Of all the pre-treatments tested 1-bromonaphthalene for 4 hours is far the best one. The rest of the pre-treatment reagents tested, 8-hydroxyquinoline, colchicine, and p-dichlorobenzene, produced chromosomes always clumped together so that counts were impossible and their morphology extremely unclear. A relatively long time of pre-treatment (more than 6 hours) was tested to accumulate as many divisions as possible but divisions could not be seen after ten hours, and chromosomes were swollen. In my investigations the material tested without pretreatment (control) did not give any results at all. After this was established by a few replications, the material was submitted always to 1% 1-bromonaphthalene. Lactopropionic orcein gave the best staining. The other stains tested induced many apparent distortations in the chromosome morphology and the cells do not have a very clear cytoplasmic background.

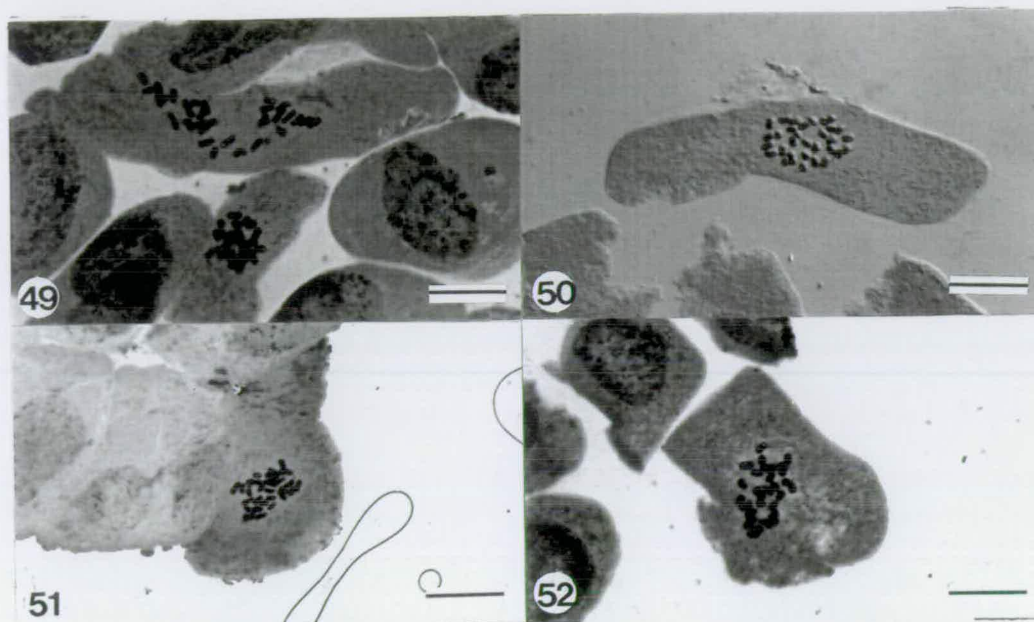
Several procedures were followed and the one described in Table 70 proved to give the best results overall. The best results were obtained using the squash method with root tips, the 1% 1-bromonanaphtalene solution for 4h at room temperature, and this pre-treatment was retained for subsequent analyses. The prefixed root tips were rinsed in running water and immersed in a fixative solution of alcohol-acetic acid (3 vol : 1 vol), for at least 24h. The fixed root tips were rinsed in running water. They were then hydrolysed for 30 min in 5 N HCl at room temperature, following which they were treated with a mixture of enzymes, and stained with orcein and squashed. Temporary slides were sealed with nail polish and stored in a refrigerator at +4.5°C in aqueous vapour chamber. The procedure for making permanent slides was that of Dyer (1963). Photographs were taken before making the slides permanent. Photographs were taken on TMAX-100 35mm film and printed on Kodakprint No. 3 printing paper.

5.3 : Observations

The number of chromosomes in plantlets raised from zygotic embryos and regenerated plantlets was counted by me. My main contribution to the cytology of *P. vera* L. was the establishment of a successful technique. The most critical technical problems in the cytology of *P. vera* L. were: (1) the number of divisions observed: this is always rather low whatever the conditions of germination, sampling of the material and the meristem used. (2) The chromosomes tend to clump together resulting in difficulties not only for the chromosome morphology, but even in counting their number; illustrations of the chromosome complement of the investigated materials are in Figs. 49-52. Figure 49 shows the chromosomes of the diploid zygotic plants after preparation with the squash method. The chromosome number of this complement was counted in more than 100 metaphase plates and confirmed that zygotic embryos of pistachio have a complement of 2n = 30. Mixploidy was never encountered. Some differences in chromosome morphology could be seen within a complement.

A chromosome count of somatic embryos of pistachio derived from EMS of immature fruit indicated that all cells were only, 2n = 30 (Fig. 50). A chromosome count of somatic embryos of pistachio derived from EMS of zygotic embryos indicated that all cells were only, 2n = 30 (Fig. 51). A diploid metaphase preparation from the EMS obtained from juvenile leaf cultures revealed all as being diploid, 2n = 30 (Fig. 52). All regenerated plantlets obtained through organogenesis had also the same chromosome number (2n = 30).The diploid chromosome numbers in all regenerated plantlets clearly indicate that there was no numerical variation in the complements of regenerated plantlets. Somaclonal variation has been detected in somatic embryogenesis of both woody angiosperms and gymnosperms (Deverno 1995). However, somaclonal variation may not always be detected during somatic embryogenic culture or during regeneration of plants. For example, abnormalities appeared after several years of growth in the field in oil

palms (Corley et al. 1986). For this reason, plants regenerated from somatic embryogenic cultures should be monitored. Somaclonal variation, however, can also be advantageous. Selection for somaclonal variants expressing important traits such as disease resistance, coupled with early, rapid screening of regenerated plants, may be a technique that can be used for improvement of woody plants (Deverno 1995). From these preliminary results it may be concluded that neither mixploidy or aneuploidy were observed in either somatic embryos or regenerated plantlets in pistachio. However, the sex of the regenerated plantlets is still questionable because those embryogenic lines produced could be a genetic male or female line.



***Figs. 49-52** : Cytological analysis (metaphase plates) of Pictacia vera L. **Fig. 49** : Metaphase plate in (a root preparation from a plantlet raised from a zygotic embryo) a root tip of germinated plantlet, bar = 18 µm. **Fig. 50** : A diploid metaphase plate obtained from a somatic embryo produced through EMS cultures of immature fruits of pistachio, bar = 18 µm. **Fig. 51** : A diploid metaphase plate obtained from a somatic embryo produced through intervening callus cultures of zygotic embryos of pistachio, bar = 18 µm. **Fig. 52** : A diploid metaphase plate obtained from a somatic embryo produced through EMS cultures of juvenile leaf explants of pistachio, bar = 18 µm.*

The resolution of chromosome morphology was insufficient to conclude whether recognisable sex chromosomes were present. If it is possible to determine sex by chromosome states, the cloned plantlets from juvenile tissues may be used in order to study clonal effects of *in vitro* sex developed plantlets. In addition it could be easier to determine the sex chromosomes, once the haploid chromosome number is obtained from anthers and pollen. No reports were made on sex determination in *P. vera* L. However, that determination will be eventually possible by improving the established technique. As far as the material is concerned, it would be profitable to explore more deeply the potentialities of the leaf meristem and ovaries and studying the best timing for sampling the material.

CHAPTER SIX

GENERAL CONCLUSIONS AND DISCUSSION

CHAPTER SIX : GENERAL CONCLUSIONS AND DISCUSSION

Methods were developed for micropropagation of pistachio, *P.vera* L. using immature fruits, leaf explants and mature stored seeds as explants through embryogenesis. An original statistical technique involving logistic regression analysis was used to analyse the probability of somatic embryo germination and embling development in each of a wide variety of experimental treatments. It is hoped that the research reported in this thesis has contributed, in a small way, to the understanding of the regeneration of mature tree material.

6.1 : Organogenesis studies

The objective of the first part of this study was to develop a method which may be used for clonal propagation of fruit-bearing pistachio trees used for commercial purposes in Turkey. In the course of this work, a method was readily established for seedlings but not for mature trees.

An effective surface sterilisation method for the production of axenic explants from *P. vera* immature seeds, mature seeds, seedlings and mature shoot tip explants was achieved. Surface sterilisation of pistachio materials was generally achieved in 20% NaOCl for 20 min and 10% H_2O_2 for 10 min. Eradication of fungal or bacterial contaminants is a major problem in mature pistachio tissue culture and has been observed by many other workers (Barghchi 1982; Bustamante-Garcia 1984 and Abousalim 1990). As this study proved, a possible measure to reduce this contamination was to reduce the size of explants cultured. It was also observed that when smaller explants were initiated, lower levels of browning were obtained.

Pistachio nodal segments of seedlings readily produced axillary shoots in all cytokinins tested whether used individually or in combination. This study revealed that BAP is more potent in terms of induction of bud and shoot number than the other cytokinins tested. Of the BAP concentrations tested, 3 mgl^{-1} BAP was the most suitable in promoting shoot multiplication of cultures, but there were no significant differences in means obtained with either 2, 3 and 4 mgl^{-1} BAP. The effectiveness of the mixture of BAP and 2iP in proliferation and in stimulating growth are also in agreement with those obtained by other workers (Barghchi 1982, Abousalim 1990). It was concluded that full strength MS medium generally supported the best growth of pistachio *in vitro,* in comparison with other media formulations used widely for tissue culture of pistachio. Abousalim (1990) also reported the superiority of MS medium for shoot proliferation of pistachio, despite the persistence of callus and faint chlorosis. Investigation into seedlings of pistachio *in vitro* clearly demonstrated that continuous illumination during culture elicits the same shoot number but a higher yield of shoot length than in complete darkness and 18 h photoperiod. The optimal light intensity for shoot initiation in pistachio seems to be in the range of 20-40 $\mu mol\ m^{-2}$

s^{-1}. The potential for inducing axillary shoots on cultured nodal segments did not change significantly with the increased age of the explanted seedlings.

Explants were obtained from 50 year-old field-grown trees of *P.vera* cultivar Antep. Meristem tip culture was found to be promising for the establishment of a culture technique because decontamination was most readily achieved with meristem tips (approximately 1mm) obtained from actively growing shoots of 50-year-old *P. vera* plants as opposed to shoot tip explants. Maximum shoot length and bud number were again obtained using the cytokinin BAP. Shoot elongation was promoted in all BAP treatments and an average of 4.8 buds per explant were obtained on MS medium containing 2.0 mgl^{-1} BAP (Table 30). The production of axillary buds has never been observed (see section 3.3). There was evidence of a significant difference in the frequencies of surviving explants obtained from explants harvested on different dates. Thus, in general the initiation of mature material was especially dependent upon the time of the year the explants were harvested and the size of explants used for culture initiation. The most commonly encountered difficulty in the micropropation of mature pistachio trees is the highly seasonal availability of the fresh experimental material and difficulty of obtaining it from Turkey. However, it is hoped that future work will establish the relationship between season of explant harvest and the establishment of rapidly proliferating shoot cultures from elite mature trees.

Initiation of roots from the axenic nodal bud segments was found to be especially dependent upon auxin, which is in accord with the usual finding that this hormone is a requirement for root induction (Barghchi 1982; Bustamante-Garcia 1984 and Abousalim 1990). The present data also indicate that the potency of auxin is significantly influenced by its type and applied concentration. Explants cultured in an MS medium containing 1 mgl^{-1} IBA and 1 mgl^{-1} NAA gave the highest rooting percentage (90%). Optimal responses were achieved with explants subcultured 10 times on multiplication media. Explants taken from 50-year-old plants were also tested for capacity to root *in vivo* following a brief immersion (10 s) in concentrated (1, 10, 100, 500 and 5000 mgl^{-1}) IBA solution but none of the cuttings rooted under greenhouse conditions. *In vitro* rooted shoots derived from *P. vera* seedlings were successfully established *in vitro* with 80% survival being attained.

Overall the previous studies and the current results indicate that the methods established for the seedlings were not effective for mature explants. Therefore, studies for the establishment of mature trees must in future be done directly on the mature plant material rather than on seedlings.

6.2 : Embryogenesis studies

In the second part of this thesis the aim was to develop an alternative method of pistachio improvement by means of embryogenesis of elite pistachio genotypes. Somatic embryogenesis *in P. vera* L. has been successfully obtained. However, there are still several limiting factors for the application of this technology to a large scale propagation. A proliferating embryogenic culture can be readily initiated from immature fruits. In the case of mature zygotic embryos and juvenile leaf explants, the percentage of initiation of embryogenic cultures is still very low. Further research is thus needed to develop embryogenic cultures from tissue explants obtained from mature trees.

6.2.1 : Embryogenic mass (EMS) induction

Initiation of embryogenic tissues of *P. vera* L. was possible from immature fruits, mature zygotic embryos and juvenile leaf explants.

6.2.1.1 : EMS induction from immature fruits

Somatic embryogenesis was induced from immature fruits of *P.vera* that were collected at three different dates. The highest response (20%) was observed from immature fruits that were harvested in mid-July. Apart from the cytokinin BAP, other tested growth regulators were ineffective in inducing embryogenic mass formation from immature fruit explants, the production of the EMS only being evident in liquid MS medium supplemented with BAP. The frequencies of EMS induction obtained here are comparable with those obtained on hybrid larch (*Larix* × *leptoeuropaea*) (Klimaszewska 1989; von Aderkas et al. 1990). The medium used and the time of the year when explanting was performed were both critical for EMS initiation. A relationship existed between the type of medium and the concentration of BAP. The responsiveness of the immature fruits to produce of EMS was not improved by using other growth regulators instead of the cytokinin BAP. This may indicate that there are specific requirements for somatic embryogenesis to occur which are not met by the majority of cytokinins. In the case of immature fruits all the material originated from one tree, but not from a controlled cross. Future work will test the controlled crosses in order to asses the suitability of media, collection date and growth regulator. The embryogenic cultures are light to deep green and may consist of numerous pre-existing somatic embryos. In the present study, the embryogenic cultures of pistachio may resemble those of conifers (Attree and Fowke 1993). They consist of somatic proembryos that may proliferate by cleavage polyembryogenesis.

6.2.1.2 : EMS induction on mature zygotic embryos and leaf explants

The mature zygotic embryos ranged in length approximately from 5 to 8 mm. Within 90-120 days of initiation, embryogenic tissue development had begun on explants. These explants produced embryogenic tissues at a maximum frequency of 16% of explanted materials (Table 67), which were greyish nodules with somatic embryos. Mature zygotic embryos appear to be the best explants for initiating embryogenic calluses since they have greater embryogenic competence than other tissue types (von Arnold 1987; Jain et al. 1988). In the present study, however, there was a significant difference in the frequencies of embryogenic callus obtained between the genotypes tested. Only three out of 8 pistachio genotypes were found to produce embryogenic callus from zygotic embryos (see 4.3.9).

Several auxins and cytokinins were tested for callus initiation from leaf explants. Leaf explants isolated from axenic germinated seeds, leaf explants dissected from regenerated shoots (from one-year old seedlings),and the regenerated leafy structures dissected from the regenerated 50-year-old trees were used as primary explants. The results obtained from callus initiation experiments demonstrated that TDZ was the most effective growth regulator for callus initiation. The friable reddish to dark green non-embryogenic callus was always obtained from the three materials tested. Only the culture of juvenile leaf tissues from axenic germinated seeds and regenerated shoots, on MS medium containing BAP resulted in embryogenic callus formation at 7, and 4% frequencies, respectively (Table 59).

Embryogenic mass development from mature zygotic embryos and leaf explants remains inefficient and the embryogenic calluses were not maintained for the production of somatic embryos.

6.2.2 : Culture maintenance

Cultures were maintained either as suspensions in liquid medium in 250 ml shake-flasks or on agar-solidified medium in Petri dishes (9 cm). Several carbohydrates (sucrose, glucose, lactose, fructose, ribose, xylose with or without BAP) at different concentrations (2, 4, 6, 8, 10 and 12%) were used in order to maintain EMS. Only four tested sugars (sucrose, glucose, lactose, fructose) had pronounced effects to proliferate EMS.

6.2.2.1 : Solid cultures

The maintenance media supporting the growth of embryogenic tissue that proliferated from different explants were different from those used in the initiation stage. Whatever medium is

chosen for the proliferation (maintenance) phase, a general concern is that not all the initiated embryogenic tissue lines are maintained in subsequent subcultures. In the case of immature fruit explants, embryogenic mass was subcultured on the same medium as the initiation medium (modified MS medium supplemented with or without 1 mgl^{-1} BAP 50 mgl^{-1} l-ascorbic acid and 500 mgl^{-1} casein hydrolysate) at 12-day intervals in the light. Once the line is established and subcutured every 3-4 weeks onto maintenance medium it remains at the proliferation stage, but a lower proliferation rate than the liquid medium maintained EMS.

Embryogenic mass development from mature zygotic embryos and leaf explants was initiated on the agar-solidified medium but remains inefficient. Embryogenic callus was subcultured on the same fresh-modified MS medium usually containing 1 mgl^{-1} BAP at approximately 4 week intervals in the light.

6.2.2.2 : Liquid cultures

Only pistachio embryogenic tissue derived from immature fruits was established as cell mass suspension. Suspension culture was initiated by transferring pieces of embryogenic tissue to liquid medium (the same as used for solid cultures but without any solidifying agent and with the addition of sucrose) in flasks placed on the shaker at 98 rpm in the light. At this time, the cultures were subcultured every 12 days by transferring 0.5 g. EMS to 50 ml fresh MS medium supplemented with or without BAP. Since EMS cultures consist only of densely cytoplasmic embryogenic cells and highly vacuolated suspensors, the dry matter in these cultures may also be directly correlated to the proportion of embryogenic cells (Schuller and Reuter 1993) and is an indicator of proliferative capacity (Onay et al. 1995b). Accordingly attention was focused on the fresh matter and dry matter content of EMSes in MS medium supplemented with different carbohydrate sources over a 12-day period. It was found that there was a positive or negative correlation between the fresh weight or dry matter content of EMS and the concentrations of carbohydrate tested. In the case of zygotic embryo and leaf explants derived callus, the cell suspensions were not established.

6.2.3 : Induction of somatic embryos

The studies described in this thesis have shown that somatic cells of *P.vera* have the ability to regenerate whole plants when placed under the appropriate culture conditions. The whole process from the induction of potentially embryogenic cells to the expression of this potential during embryoid development and plantlet regeneration involves a complex series of changes in gene expression which determine the metabolic and physiological activities of the cells.

The induction and formation of somatic embryos may be either direct or indirect depending on whether there is an intervening callus stage. Direct embryo formation is from cultured explants and indirect formation from callus tissue, cell suspension and protoplast cultures. A mechanism of induction of embryogenesis *in vitro* has been put forward by Williams and Maheshwaran (1986). They have postulated that in the young zygotic embryo many cells are determined to follow embryogenic development. As the embryo matures and germinates, the number of cells that can form somatic embryos decreases. Consequently, the capacity to form somatic embryos decreases. This hypothesis predicts that direct induction of somatic embryos proceeds from cells which have already been determined for embryogenic development prior to placement in culture, requiring only favourable growth conditions to allow them to proliferate and express their embryogenic potential. In contrast, the induction of indirect somatic embryogenesis requires the re-determination of differentiated cells via callus formation, and then the induction of an embryogenically determined state.

The two pathways of somatic embryogenesis in *P. vera* were reported in this thesis, i.e., via EMSes from immature fruits which is an example of direct embryogenesis. The de-differentiation of explanted material (i.e., mature zygotic embryos and leaf explants) occurs during callus proliferation in the presence of 2,4-D and TDZ, respectively, while re-determination of some of the cells to an embryogenic state within the callus tissue is achieved by subculturing relatively undifferentiated calli to a BAP containing media (zygotic-embryo-derived callus or leaf-derived callus).

6.2.4 : Effects of growth regulators

As shown in this thesis, the plant regulators are the key external factor in inducing somatic embryogenesis in EMS or embryogenic callus cultures of *P.vera*. The cytokinin BAP, the synthetic auxin 2,4-D and the cytokinin-like TDZ were essential for the induction of EMS or callusing tissues from explanted materials (immature fruit, zygotic embryos and juvenile leaf explants, respectively). The cytokinin BAP may play a direct role in producing embryogenic tissues in explanted pistachio materials. It may be noted that the physiological state of the explanted tissues has a great influence on the subsequent pattern of differentiation, since the immature fruits induced EMS directly after culturing in liquid MS medium. Although morphologically alike, calluses derived from zygotic embryos and juvenile leaf tissues show some differences in their responses to growth regulators. Once the transfer of zygotic embryos or leaf derived callus from a 2,4-D or TDZ primary culture to a BAP secondary culture, has been made both type of calli turn into a compact green EMS. This clearly indicates that the state of the tissue or callus, which may have been determined at the point it was induced, plays a key role in deciding the way in which it responds to BAP. From these, it is apparent that the morphogenetic

response of the callus is a result of the ability of the interaction between the tissue or callus to differentiate, and the effect of the cytokinin BAP. Few reports are made on the control of somatic embryos using the cytokinin BAP (Norgaard and Krogstrup 1991). Structure of common natural and synthetic cytokinins are adenine derivates (Salisbury and Ross 1992). Fosket and his colleagues (Fosket 1977; Fosket et al. 1981) found that cytokinins that promote division of cells in tissue culture by increasing the transition from G_2 to mitosis and that they do this by increasing the rate of protein synthesis. I guess some of these proteins induced by the cytokinin BAP could be enzymes or structural proteins needed for embryogenesis. There is as yet no explanation as how embryogenesis is started by the cytokinin BAP or other growth regulators and no special enzyme or other protein that might lead to embryogenesis has been discovered in cells treated by BAP or any growth regulator.

6.2.5 : Maturation of somatic embryos

Several media and growth regulators in agar-solidified or liquid MS medium (auxins, cytokinins and ABA) together with sucrose were tested to promote the development of high quality somatic embryos. The formation of high quality SEs was dependent upon the presence or absence of growth regulators in the maturation treatment depending on the origin of EMS. In the case of immature fruit-derived EMS, in treatments with BAP the somatic embryos germinated precociously, whereas in treatments with ABA (0.25-2 mgl^{-1}), all somatic embryos developed slowly and remained non-germinating (Onay et al. 1995a). The formation of high quality SEs was also dependent upon the media used for maturation. Further development of embryos and the regeneration of young plants from embryos also requires the transfer of mature SEs from liquid MS medium to agar-solidified MS medium. Abnormal somatic embryo development was often evident when the embryos matured in agar-solidified MS medium together with ABA or BAP (Onay et al. 1995a). Contrary to these results, abnormal somatic embryo development in carrot (Ammirato 1984), Norway spruce (Becwar et al. 1989), and interior spruce (Roberts et al. 1990) is known to be suppressed by ABA.

In sections 4.1.5.5 and 4.1.5.6, ABA or BAP were added only during maturation and EMSes were obtained from liquid medium without growth regulators. Neither germination nor plantlet development medium contained BAP or ABA. In fact, treatments with ABA or BAP were unnecessary for the formation of individual embryos and elongation of globular embryos because the EMSes cultured in liquid MS medium produced more somatic embryos than BAP and ABA applied treatments. This finding is in contrast to the reports of Boulay et al. (1988) for *Picea abies* who found that treatment with ABA was necessary for the formation of individual embryos and elongation of globular embryos.

In pistachio, the sucrose and ABA or BAP interaction for the maturation of somatic embryos was statistically significant. This was also found in some conifer species (Finer et al. 1989, Cheliak and Klimaszewska 1991). Variation in pistachio somatic embryo maturation was observed not only among the different growth regulators, but also among different media tested. However, the number of somatic embryos matured in liquid MS medium was higher than any other tested treatments. The growth regulator-free liquid MS medium realised the highest embryogenic potential (208 somatic embryo per gram of EMS), whereas for the BAP supplemented medium it was 206). In general, EMS does not require growth regulators in the culture medium to develop into somatic embryos. Therefore, these results suggest that there are pre-existing embryos in the EMS induced from immature fruits of pistachio and they could be matured in liquid MS medium without a growth regulator. However, none of the transferred callus or EMSes derived from mature zygotic embryo and from leaf explants on to growth regulator-free MS medium showed somatic embryos. As a result of the above study it can be concluded that pistachio somatic embryos can be matured in liquid or agar-solidified medium with or without ABA and BAP. The number of SEs on 0.5 mgl^{-1} BAP and the control treatments (without the growth regulators) with sucrose concentrations 2% and 6% was significantly higher ($P < 0.05$) than on the remaining treatments (Table 43), respectively. Media with 0.5 mgl^{-1} BAP and 2% sucrose induced the most embryos (50.1 per 250 mg fresh weight) (Table 43). Media with 0.5 mgl^{-1} ABA and 4% sucrose induced the most embryos (50.7 per 250 mg fresh weight) (Table 44). Similarly, the development of the somatic embryos of *Picea abies* using a liquid culture maturation phase has also been reported (Boulay et al. 1988), *Picea glauca* (Attree et al. 1994), *Picea mariana* (Tautorus, 1990), *Picea sitchensis* (Krogstrup, 1988),and *Pseudotsuga menziesii* (Durzan and Gupta 1987). Cultures maintained on agar-solidified medium produced somatic embryos as much as on liquid medium. These results suggest that maturation may be achieved in liquid or agar-solidified media but maturation on agar-solidified was longer than in liquid medium, and germination and plantlet development was highest when cultures had been grown in liquid medium.

In the case of zygotic embryo and leaf explants originated embryogenic tissues, embryos developed on a modified MS medium supplemented with casein hydrolysate, l-ascorbic acid and generally 1 mgl^{-1} BAP. Embryos were further elongated and developed to a cotyledonary stage on a filter support containing agar-solidified MS medium without growth regulators.

Overall, the maturation of somatic embryos in pistachio depends on a number of factors and their interactions. This study revealed four of them (origin of EMS, sucrose and presence, and absence of growth regulators) and more importantly, the interaction among them.

6.2.6 : Germination and plantlet development from somatic embryos

Germination and development of plantlets from somatic embryos of pistachio was affected by many factors. Appropriate treatments with ABA or BAP, followed by a liquid or agar-solidified maturation treatment, are two critical factors that lead to high germination rates. These can be further manipulated by maturation time. With *P. vera*, a 40 day treatment promoted root emergence to an average of 0.54, as compared to 0.19, 0.29 and 0.43 for 10, 20 and 30 day treatments, respectively (Onay et al. 1995c).

In this study the highest germination frequency was obtained with somatic embryos matured in the absence of ABA or BAP. This is in agreement with the results reported by Ammirato (1984). The plant growth regulator-free medium gave rise to the developmentally highest frequency of germinating (0.62) and developing plantlets (0.52) (Tables 52 and 54 respectively).

Germination and plantlet recovery were found to be reliable indicators of the optimal or suboptimal maturation treatments of pistachio somatic embryos. The highest germination frequency was obtained when 2 weeks maturation treatments were applied without plant growth regulators. The treatment of 2 weeks seemed to be more beneficial than 3 weeks without plant growth regulators. Morphological changes of the somatic embryos were observed during the third week of maturation where they enlarged and turned dark green. When the cultures were kept for 4 weeks in liquid medium, then transferred to a germination medium, a few somatic embryos germinated. As with germination frequency, plantlet recovery was affected by the time of maturation, with 2 weeks being superior in respective to the ABA or BAP concentration used (Table 51 and 53). In pistachio, it appears that embryo germination did not require growth regulators, since few of the somatic embryos transferred on to growth regulator MS medium developed health looking plants. Embryo germination is clearly a complex process controlled by at least three factors media, growth regulators and maturation time. To achieve the most efficient maturation of somatic embryos we have to combine all these factors in order to fully exploit the embryogenic potential of various pistachio materials. However, in the case of two embryogenic lines (induced from zygotic embryos and leaves) although BAP was effective for embryo development, further development of somatic embryos was poor.

The optimum concentrations of the main constituent giving the highest probability of plantlet development were obtained with 2 mgl^{-1} BAP together with 8% (w/v) sucrose concentration after 14 or 21 days of culture and 6 weeks of maturation period on agar-solidified medium (Onay et al. 1995c). However, the optimum concentrations of the main constituent giving the highest probability of plantlet development were obtained with 0.5 mgl^{-1} BAP together with 4 or 6% (w/v) sucrose concentration after 40 days of culture and 2 weeks of maturation period in liquid MS medium (Table 54). Therefore, using the liquid MS medium for the maturation of somatic

embryos produced not only higher recovered plantlets but also reduced time for the regeneration plantlets. In the case of zygotic embryos and juvenile leaf explants, somatic embryogenesis and plantlet regeneration were possible although propagation was still very poor.

6.2.7 : Statistical analysis

For the first time, an original statistical technique involving logistic regression analysis based on GENSTAT, has been applied for the analysis of the probability of somatic embryos' germination and embling development date. The developed statistical model is submitted for publication (Onay et al. 1995c). In the current study, analogous models were made for the analysis of the effects of the BAP, ABA and sucrose on germination and plantlet development data. The response variables, the number of somatic embryos germinated and the number of plantlets developed, are binomially distributed. Therefore a linear logistic regression analysis, which avoids transformation problems, is used to analyse the data. Analysis of deviance tables and the fitted probabilities of germination and plantlet development are presented by different factors to draw sensible conclusions from the analysis. From the tables obtained, one can readily see the optimum conditions of the multivariate treatments tested.

6.2.8 : Encapsulation of somatic embryos and embryogenic mass

The use of somatic embryogenesis for the cloning of superior genotypes generates a need for long term preservation during field evaluation of clones, since the culture value of EMS or cell lines is unknown, owing to their juvenile origin. Encapsulation is a practical procedure for short-term storage of embryogenic pistachio tissue, and may be applicable to the preservation of desirable elite genotypes. This research is in its infancy but it has been shown that mature somatic embryos of *P. vera* can be encapsulated in calcium alginate gel and still retain a modest level of germination capacity *in vitro* (Onay et al. 1995b). However, it remains to be established whether the storage period can be extended further than the 60-day period. Although the conversion frequency of completed plantlets from synthetic seeds of pistachio was low (14%), the results obtained were positive, demonstrating that the method is feasible, and suggested that it may be possible to optimise this technique to improve the rate of production of complete plantlets after the storage of embryos.

6.2.9 : Cytological evidence

So far the chromosome reports in this species (*P.vera* L.) have been made by a few workers (Zohary 1952, Maggs 1973 and Barghchi and Alderson 1989). However, none of them provided

any evidence in their publications. More to that point, they have reported different chromosome numbers. The chromosome of the diploid zygotic seeds was counted after preparation with the squash method. The chromosome number of this complement was counted in more than 100 metaphase plates and confirmed that zygotic embryos of pistachio have a complement of 2n = 30. The number obtained can hardly throw any light on the phylogeny of the species. A cytological data with an unclear morphology have been reported for the first time. Therefore, further investigations is essential for the actual chromosome number of *P. vera* L.

To confirm the origin and nature of the micropropagated plantlets, the chromosome numbers were determined. The root tip was pre-treated overnight with 1% α-bromonaphtalene, acid hydrolysed and stained for a few minutes in 2% acetic orcein. The chromosomes were counted in several regenerated plantlets. All regenerated plantlets via embryogenesis or organogenesis had also a complement of 30 chromosome (see Figs. 49-52). The diploid chromosome number in all regenerated plantlets clearly indicates that there was no numerical variation in the regenerated plantlets. Somaclonal variation has been observed among woody plant regenerants. The subject has been reviewed by Ahuja (1987). Lester and Berbee (1977) observed variation in height, number of branches, leaf traits, and chromosome number among callus derived plants of *Populus nigra* and *P* x *euramericana*. Many traits have been unidentified from somaclonal variation studies (Morrison et al 1988) but few of these have been analysed genetically. Genetic analysis can be slow and time consuming, particularly for woody tree species. Therefore, its nature has not been explored for woody plants.

6.3 : Growth of plantlets in the greenhouse

Strikingly uniform growth was observed for the regenerated plantlets (via organogenesis or embryogenesis). Environmental conditions were not conclusive to good growth. Pistachio trees need dry atmosphere and high light. Watering requirements are not fully understood. Pistachio trees prefer a deep root run. Therefore, the plantlets produced were frequently repotted. Plants do not like to be disturbed. They were also prone to infestations of whitefly and red spider. The materials propagated have not been planted onto a typical pistachio regeneration site in Edinburgh. Therefore, the growth characteristics may not be thoroughly obtained.

6.4 : Future prospects

Based on the presented information in this thesis, although somatic embryogenesis seems very promising in achieving large scale production of pistachio plantlets, the following outlines some of the problems commonly associated with plant tissue culture, and further research required:

1. Because of the limited conditions, the morphogenetic ability of mature trees explanted throughout the year was not tested. To micropropagate mature trees, it is necessary to investigate further times of the year for explants to be harvested.

2. Efforts should be made to improve and extend the basic knowledge of somatic embryogenesis initiation using a model system developed for immature fruits. This is essential because the study of a detailed examination of the biochemical and physiological changes accompanying somatic embryogenesis depends on the production of sufficient quantities of somatic embryos of different developmental stages. This would rely on the establishment of the embryogenic cell masses or suspension culture for the production of large numbers of developing embryos under controlled conditions similar to that already achieved with pistachio EMS cultures. Also, a technique of planting out free cells in a layer of agar medium to produced clones of single-cell origin would allow a more precise analysis of the embryogenic potential of the cells in culture.

3. Growth of regenerated plants via organogenesis or embryogenesis in the greenhouse remains a formidable problem. For acclimatisation of axenic plantlets, the usefulness of mycorrhizal associations in the formation and growth of roots should be studied. It is also necessary to implement large-scale and long duration field tests of emblings in comparison with seedlings.

4. Most of the investigated materials have been obtained from juvenile tissues, particularly immature fruits, zygotic embryos and leaf explants. The ability to produce embryogenic tissue was depended on not only a growth regulator but also on the age of explants used. Tissue from mature trees did not produce embryogenic tissue. The successful induction of somatic embryogenesis provides the basis for further studies on biochemical and physiological aspects of this *in vitro* morphogenesis. Attempts should be made to establish biochemical and molecular markers to identify juvenile characteristics of embryogenic tissues and to facilitate studies on the induction and development of somatic embryos from mature tissues.

5. High levels of secondary metabolites may be anticipated because of the observed effects of cellular organisation in somatic embryos. A developed system for the production of somatic embryos could theoretically produce a continuous supply of the metabolite throughout the year.

6. Attempts for callus production and regeneration of plants from cultured inflorescence of different *P.vera* varieties should be made.

7. In the future, propagation of *P. vera* may include the combination of somatic embryogenesis and cryopreservation. EMS can be induced from immature fruits of elite genotypes and cryopreservation for the time required for early field trials. It permits the maintenance in a juvenile status of future elite genotypes while the cloned plantlets are being tested in the field. Therefore there is a need for the long term preservation of EMS. However, once elite-mature trees are successfully rejuvenated cryopreservation may be bypassed.

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