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Lahore Laboratories

FIRST SIX MONTHLY REPORT ON
THE PROJECT
"REGENERATION OF PISTACHIA vera"
Grant No.936-5542-G-00-7219-00

LAHORE LABORATORIES
Pakistan Council of Scientific & Industrial Research
SHAH RAH-E-JALALUDDIN ROOMI, LAHORE-16

Subject: FIRST SIX MONTHLY REPORT ON THE PROJECT "REGENERATION OF PISTACHIA vera" GRANT NO.936-5542-G-00-7219-00

Work on the above mentioned project was initiated on 1.10.1987 after appointing the following staff:

1. Mr. Syed Tariq Iqbal Shah, Research Geneticist
2. Mr. Zaheer Ahmad, Research Chemist
3. Mr. Syed Zamurd Hussain, Junior Experimental Officer
4. Miss Fehra Zafar, Junior Experimental Officer
5. Mr. Syed Mujtaba Gilani, Laboratory Assistant
6. Mr. Naveed Haider, Laboratory Attendant

The first priority was to get facilities lined up. In this context work on fabrication of green house was initiated. One unit measuring 22' x 28' with fiber glass covering, fully air-conditioned and with humidity and light control measures is fully operational. Another unit with double glass insulation is nearing completion. It will also be equipped with all the facilities available in the fiber glass unit.

Quotations for equipment, chemicals and apparatus, in US$ were also invited and discussed with USAID representative in Pakistan. A final proposal for purchase of these times is ready and will be submitted to the USAID.

3 workers (laboratory as well as project workers) have got themselves registered for their Ph.D. degree with the University of Punjab. Copies of their synopsis are attached.

Progress of work conducted during the last six months is also appended.

The delays in the finalization of purchases of equipment have adversely affected progress of work. It is hoped that the proposal submitted to the USAID for purchase of equipment and other items would be finalized soon and the items would arrive during the next six months.

Fiscal report and requirement for the next six months are also attached.

Seeds of P. vera (with and without seed coat) were disinfected, before germination, by soaking in mercuric chloride solution (0.1 %). It is evident, from table 1 that soaking for 10 minutes was most suitable for controlling contamination during germination. Only 10 % explants became infected, whereas germination response was fairly good. The seeds with seed coats took 15 minutes for disinfection. The rate of contamination was some what more i.e. 20 % but there was improvement in the germination rate. The drawback in either case was that health of the aseptically cultured seedling was quite poor.

The effect of disinfectants on the growth of the seedlings and their health is presented in table 2. The seed coats seems to maintain some compression force against the walls of cotyledons which was indirectly transferred to the germinating embryo. As a result of this, water absorption was slow and the growing plumule gradually emerged out of cotyledons without damaging cotyledonary stalks. On the other hand seedlings germinated from seeds without seed coats broke one of the cotyledonary stalks. Although such seedlings grew vigorously in the initial stage but dependence on one cotyledon soon affected their growth and vigor. Uninterupted enlargement of cotyledons due to excessive water absorption, in case of seeds with seed coat, caused minute injuries alongwith abaxial surfaces of the cotyledons which resulted in browning of the cotyledons and of the medium, and adversely affected growth of the seedlings.

Improvement in germination percentage and health of the seedlings which emerged from seeds grown on the media supplemented with various concentrations of BA, NAA and/or KIN is depicted in table 3. A slight increase in the rate of germination from 40 - 42 % was noticed when the seeds were propagated in 1.0 mg/l BA medium alone or a combination of 0.1 ml/l BA and 0.25 mg/l NAA. 47 % germination was observed in a medium containing 1.0 mg/l KIN and 0.25 mg/l NAA, whereas a combination of 4 mg/l BA, 1 mg/l NAA and 1 mg/l KIN further enhanced the germination percentage to 55 %.

There was a marked increase in the size of the leaves and diameter of main shoots of seedlings grown on medium supplemented with hormone (Fig.1). In case of media containing BA alone, BAA + NAA or KIN + NAA single shoots were formed and size of the diameter was approximately 10, 15 or20 mm respectively. Multiple shoots with 25 - 30 mm length were induced by the presence of BA + NAA + KIN. The area of the leaves was four times more than those of the seedlings grown on hormone free medium (table 3).

Explants of seedlings, root, shoot and cotyledons cultured on media containing different compositions of BA and NAA exhibited different morphological behaviour (table 4). 1 mg/l each of NAA and BA induced callus formation in explants of cotyledons, cotyledonary stalk (fig.3) and roots. Cotyledons formed adventitions roots (fig.4) on medium containing 0.1 mg/l BA + 1.5 mg/l NAA while globular embriods

were observed in explants of cotyledons propagated on the medium containing 2.0 mg/l BA and 0.1 mg/l NAA.

Dominance of apical shoots was common among the seedlings. Shoot tip necrosis (fig.5) appeared at the age of 3-4 weeks in almost every seedling. Proliferation of the axillary buds was noticed with the setting in necrosis of the main shoot apices.

Phenomenon of shoot tip dying due to necrosis and proliferation of axillary shoots appeared alternately. Development of techniques to overcome problems of shoot tip necrosis and browning of explants is included in the future programme.

Table 5 shows excerpts from a series of experiments conducted on juvenile explants of Pistacia vera to obtain friable calli suitable for suspension culture. Effect of various growth regulators added singly or in combination on the rate of callus formation and texture was also studied.

TABLE 1

Effect of mercuric chloride treatment period on effective disinfection of seeds

Nature of seeds	Soaking Time min.	Contamination %	Germination response	Remarks
Without seed coats	3	100	+	Germination accompined with fungal contamination; not fit for *in vitro* culture
	10	10	+++	Contamination controlled; befitting *in vitro* culture
	15	Nil	-	Seeds died
With seed coats	10	70	+++	Germination restricted due to fungal attack; unfit for *in vitro* culture
	15	20	+++	Suitable for *in vitro* culture
	20	10	+	Germination is delayed/seeds die off

+ Poor
+++ Fairly good
- Nil

TABLE 2

Effect of inoculating P. vera seeds with/without seed coats on germination

	Seeds with seed coats	Seeds wihtout seed coats
Germination period	2-3 days	2-3 days
Seedling growth	Normal	Vigorous in early stages
Mechanical injury	No signs	Contact of seedling with one of the cotyledon is broken.
Browning of Tissue	Colouring substrate are discharged from seed coats which do not inhibit normal growth of seedling	1) Cotyledons also grow with seedlings. 2) Vigorous increase in size of cotyledons without seed coat protection causes numerous minor injuries on their abaxial sides. 3) As a result polyphenols were discharged which were injurious to plant health.

Table - 3

Effect of growth regulators on germination rate and seedling vigor

GRs	Concentration mg/l	Germination	Shoot		Leaf size LxW mm
			Thickness mm	Number	
BA	1.0	42	10	Single	10:7
BA + NAA	1.0 0.25	42	15	Single	15:10
KIN + NAA	1.0 0.25	47	20	Single	25:15
BA + KIN + NAA	4.0 1.0	55	30-35	Multiple	40:30

Table - 4

Effect of various concentrations of BA and NAA on morphological response of P.vera seedling explants

BA		NAA	EXPLANT	Morphological response
1mg/l	+	1mg/l	Cotyledon	Callogenesis
			Cotyledonary stalk	Rhizogenesis
			Root	Callagenesis
1mg/l	+	0.1	Cotyledonary stalk	Callagenesis
0.1	+	1.5	Cotyledon	Rhizogenesis
2.0	+	0.1	Cotyledon	Callogenesis

Table - 5

Effect of various combinations of GRs on induction and friability of callus formed by *Pistacia vera* seeds

Medium No.	Growth Regulators (mg/l)							Callus characteristics	
	2,4-D	IAA	NAA	IBA	KIN	BAP	GA3	Induction	Texture
9	2.0	-	-	-	2.0	-	-	+++	++
15	1.0	-	-	-	-	-	-	+++	+
16	2.0	-	2.0	-	2.0	-	-	+++	+++
17	0.5	2.0	-	-	0.5	-	-	+++	++
23	0.2	-	-	5.0	-	5.0	0.5	++	+++
30	3.2	-	-	-	-	-	-	+++	+++
34	4.0	-	-	-	-	-	-	++	+++
41	-	2.0	-	-	-	-	-	++	+

+++ Very good

++ Good

+ Fair

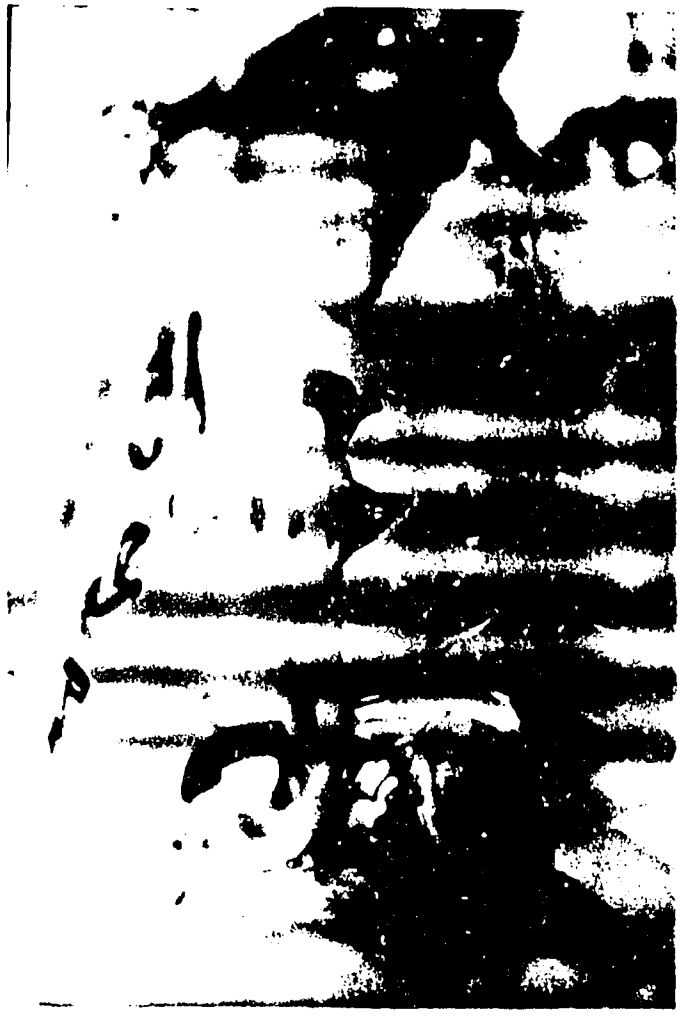


Fig.1: A four weeks old healthy seedling of *Pistacia vera*.

Fig.2: Multiple shoot formation in *Pistacia vera* at seedling stage.

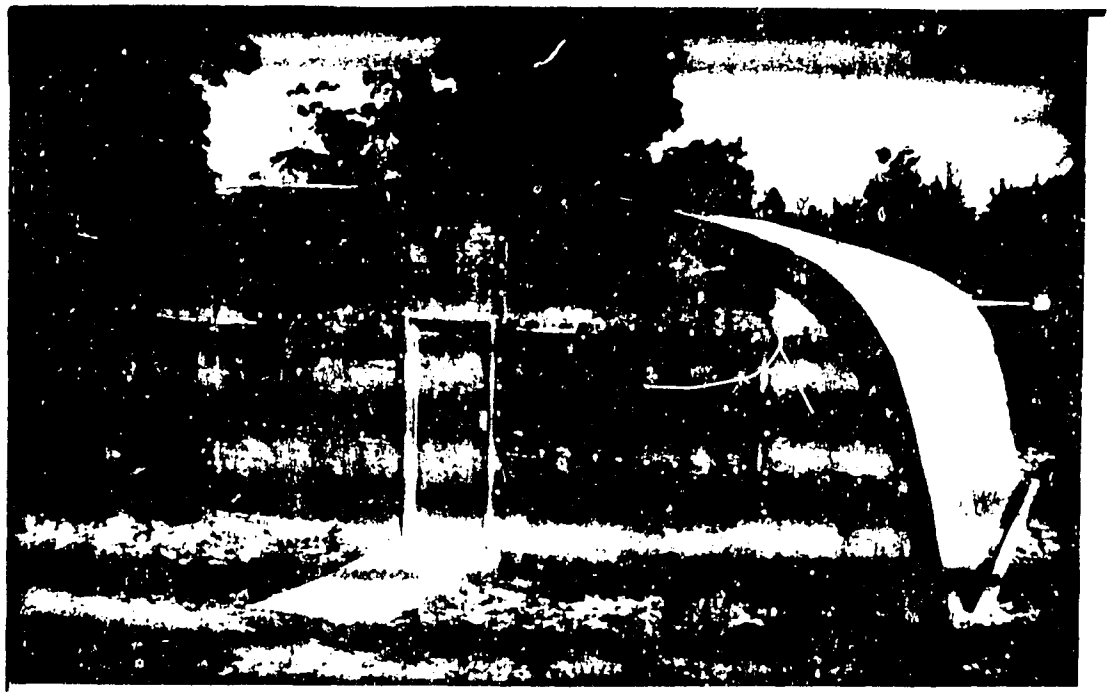
Fig.3: Callus formation on cotyledonary stalks of *Pistacia vera*.

Fig.4: Adventitions root regeneration from cotyledons of Pistacia vera.

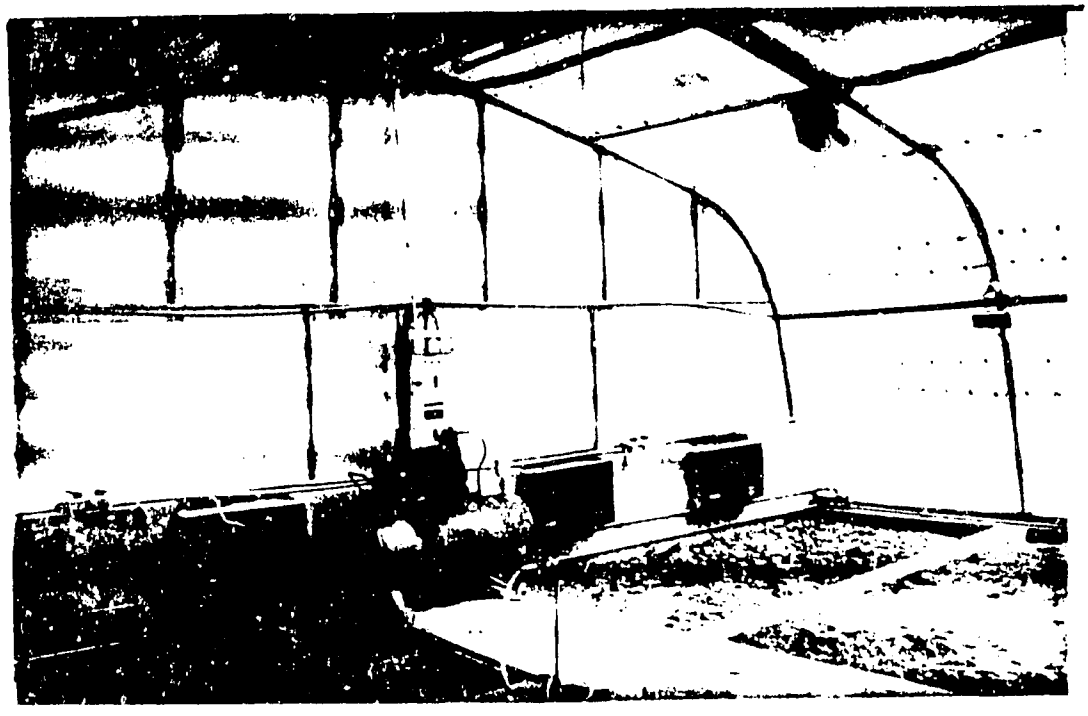
Fig.5: A six weeks old nacrotic seedling of *Pistacia vera*.

Green House No.1

Fibre Glass Construction



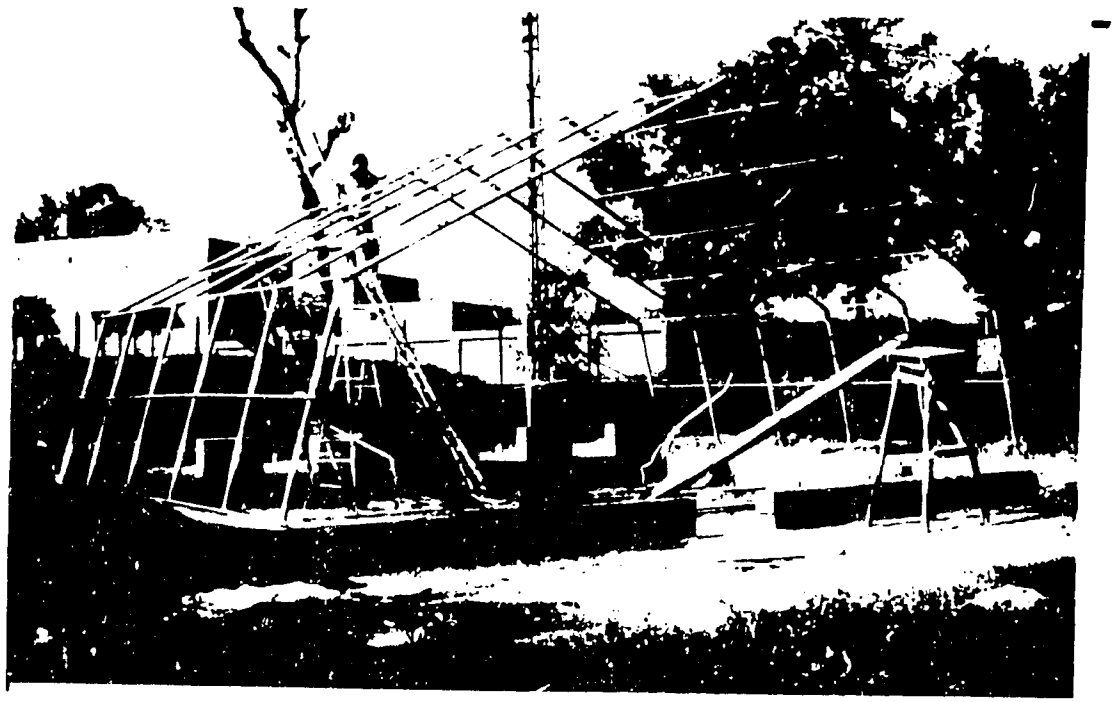
External view



Internal view

Green House No.2

Glass Construction



Overall view of under-construction green house.

FISCAL REPORT

Head of account	Amount allocated (Rs.)	Amount spent (Rs.)	Allocation for the 1st year ($)	or (Rs.)	Demand for the next six months (Rs.)
Salaries	33,000.00	54,393.08	9,721	1,65,257.00	80,000.00
Local travel	7,000.00	3,780.00	2,500	42,500.00	25,000.00
Miscellaneous	3,500.00	6,688.35	2,000	34,000.00	30,000.00
Green House	5,00,000.00	2,00,427.97	31,250	5,00,000.00	2,99,572.00 (likely to be spent)
Glass apparatus	5,000.00	5,500.00	2,500	42,500.00	37,500.00
				Total:	4,72,072.00

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Amount obtained for 1st six months	=	Rs. 5,48,500.00
Total amount spent	=	Rs. 2,70,789.40
Amount left in bank	=	Rs. 2,77,410.60
Demand for the next six months	=	Rs. 4,72,072.00

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Requested	=	Rs. 4,72,072.00
In hand	=	Rs. 2,77,410.60
Demand	=	Rs. 1,94,661.40

Note: Most of the facilities in term of chemicals and equipment were provided by the laboratories. This was the reason for savings under various heads.

A) <u>Facilities not available</u>

1) Most of the laboratory equipment enlisted on PD-7/8 of contract copy are awaited.

2) Chemicals to be purchased from project funds.

3) Full strength man power.

B) <u>Facilities available</u>

1) Inoculation chamber equipped with UV light.

2) Light and temperature controlled growth chambers.

3) Chemicals for media preparation.

4) Space for development of a sophisticated laboratory and growth chambers.

C) <u>Future Programme</u>

1) Availability of chemicals and equipment is anticipated in the next six months.

2) Air conditioning of the laboratory and growth chambers with split type AC will be accomplished.

D) <u>Permission to disburse the money</u>

Funds saved under different heads may please be allowed for purchase of books and journals and for creation of other facilities for proper execution of the research work mentioned in the project.

FEDERAL CASH ADVANCE STATUS REPORT
(Report Control No.W-245)

A. Period covered by this report:

FROM (Month, day, year) October 1, 1987

TO (Month, day, year) March 31, 1988

Period covered by the next report:

FROM (Month, day, year) April 1, 1988

TO (Month, day, year) September 30, 1988

B. Cash Advance Use and Needs:

1. Cash advance on hand at the beginning of this reporting period..... Rs.5,48,500.00
2. U.S. Treasury check advance(s) receiving during this reporting period..... Rs.5,48,500.00
3. Interest earned on cash advance during this reporting period..... Nil
4. GROSS cash advance available during this reporting period (Lines 1,2 & 3)..... Rs.5,48,500.00
5. LESS, interest remitted to AID during this reporting period..... Nil
6. NET cash advance available during this reporting period (Line 4 minus line 5)..... Rs.5,48,500.00
7. Total disbursements during this reporting period including subadvances (see footnote 1)..... Rs.2,70,789.40
8. Amount of cash advances available at the end of this reporting period (line 6 minus line 7)..... Rs.2,77,410.60
9. Projected disbursements, including subadvances, for the next reporting period (see footnote 2)..... Rs.4,72,072.00
10. Additional cash advance requested for the next reporting period (line 9 minus line 8)..... Rs.1,94,661.40
11. Total interest earned on cash advance from the start of the grant to the end of this reporting period, but not remitted to AID.... Nil
12. Total cash advances to subgrantees, if any, as of the end of this reporting period Nil

FOOTNOTES

1. The grantee shall submit a cumulative detailed report of disbursements by BUDGET line item quarterly.
2. The grantee shall attach a summary, by BUDGET line item, of its projected disbursements for the next reporting period.

CLONAL PROPAGATION OF PISTACHIO THROUGH TISSUE CULTURE

SYNOPSIS FOR DOCTORAL WORK

IN

BOTANY

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21

CLONAL PROPAGATION OF PISTACHIO THROUGH TISSUE CULTURE

Pistachio nut is a deciduous diocious medium sized tree crop. It belongs to family Anacardaceae. Pistachio nut requires long warm days summers for good cultivation and enough cold winters to break their dormancy. The pistachio tolerates well drained saline soils. Prolonged summer rains and high humidity at or near harvest time are determental to quality nut production. In Pakistan its cultivation is restricted to cold dry regions of Baluchistan i.e. Quetta and Kallat, Skardu in Azad Jammu and Kashmir and Dera Ismail Khan in N.W.F.P. (Baluch 1979).

Pistachio is represented in the world by 10 species, among them 4 are present in Pakistan (Nasir 1983)

1. *Pistacia chinensis*
2. *P. khinjik*
3. *P. atlantica*
4. *P. vera*

the last one is edible.

Traditionally *Pistacia* is propagated both sexually by means of seedling and asexually by budding or grafting (Khan 1981). Due to extremely heterogeneous nature of *Pistacia* usually asexual propagation is preferred. The

later one is also adapted to maintain the good quality plants. So far establishment of new orchard on industrial basis has failed to develop at national or international level (Optiz 1975). Optiz has described some of the problems.

1. Lack of suitable pollinators.
2. The long time often required for an orchard to come into commercial production (8 to 15 years).
3. Inadequate nursery stock.
4. Failure to develop uniform high producing orchards.
5. Erratic production - "alternate bearing"

In Pakistan following short comings are encountered:-

Mostly trees are derived from wild stands, hence

1. Fruit yield is low.
2. Kernel size is smaller than desirable.
3. Often nuts are seedless.
4. Tolerance of trees to soil and environmental stressess resistance is low, and diseases due to microbes, as well as, pests.
5. Ratio of male to female plants is 10 - 12 : 1

All the above cited problems can be solved with the help of tissue culture techniques. Tissue culture technology has been developed in many fields after nineteen fiftees. Now it is a powerful agricultural tool which enables the plants to combat against degenerative genetic and ecological trends.

The term tissue culture is a general term which covers the cultivation of plant parts whether an organ, a group of cells, a single cell, or even the protoplast within a defined environment. According to "the cell theory" presented by Schwann (1839) individual cells of a multicellular organism are capable of developing independently if provided with the proper external conditions. Later on this phenomenon was named Toti-potency by . Morgan (1901) where cells can form large and uniform population of cells which can be converted en masse to roots, shoots or embryo like structures. On the other hand by imposing upon such cells the conditions to which they are subjected within the meristem of the intact plants and thereby converting them to specialized cells.

So far plant tissue culture has been proven to be a strong biotechnical tool to improve means of

plant propagation, plant health and plant breeding up to satisfaction.

Up till late seventies, tissue culture practices were mostly confined to herbaceous genera. The methodology developed for mass propagation of strawberry (Boxus et al 1977) provoked the idea of developing economically feasible commercial production of woody plants. Woody genera are present in all the four large groups of plants categorised on the basis of human interest and tissue culture practice.

1. Agriculture
2. Ornamental
3. Medicinal
4. Forest

Most of the applications of tissue culture propagation of woody plants in the past decade have been with horticultural crops. Initiation of large scale production of apple and peach (Abbott & Whitely 1976) root stocks in vitro was major tissue culture methods applied to woody horticultural crops.

During the same period meristem tip culture techniques were developed for a number of woody fruit bearing plants.

Prunus	(Boxus and Quoirin 1974)
Peach	(Zuccherelli 1979; Zuccherelli *et al* 1978)
Cherry	(Zimmerman 1980)
Plum	(Jones & Hopegood 1979)
Apple	(Loreti & Morini 1982)
Red Respberry	(Anderson 1980)
Black berry	(Broome & Zimmerman, 1978, Harper 1978)
Walnut	(Driver & Kuniyuki 1984)

In early eightees tissue culture techniques have been introduced to *Pistacia*. Al-Barazi and Schwabe (1982) did rooting of soft wood cuttings of adult *Pistacia vera*. A year later Barghchi and Alderson (1983) reported he *in vitro* propagation of *P. vera* from seedling tissues. Proliferated shoot cultures established from shoot tips and nodal buds segments excised from aseptically germinated seedling were rooted *in vitro*. The rooted shoots were than transferred to soil for establishment.

The same group of workers (Barghchi and Alderson (1985) achieved rooting of proliferated shoot apices and nodal buds of *P. vera*. L. and commercial cultivars of Ohadi and Kalleghochi adapting the similar procedure as reported earlier.

Al-Barazi and Schwabe (1985) investigated juvenility effect and genetic differences in relation to rooting by testing extractable substances obtained from P. vera. Inhibitory effect were not detected in extracts of difficult to root mature pistachio. However, a technique of shoot/root grafting resulted adventitious root formation on cuttings of pistachio.

At present the aim of study is to save the endangered pistachio plantation of Pakistan. Therefore, it is desired to select healthy cultivars of local Pistacia varieties and utilize their explant for clonal propagation through tissue culture. The rooting of proliferated shoot tips and nodal buds of seedling and 2 years old plants have been achieved. Barghchi and Alderson (1985) On the other hand graftage of shoot and root has resulted only in the formation of adventitious roots. But there are few draw backs in utilization of seedling and young sampling tissue explant.

1) There is least information about the phenotype expression of seed borne parent plant.

2) Disease resistant variety of a high yielding plant can not be produced until plant is grown up and culture indexing has been done previously.

From stand point of tissue culture of mature Pistacia tree explant, Zimmerman (1985) has pointed out the difficulty in culture establishment of Pistacia. In light of Franclet,'(1980) review on "rejuvenation" of woody plant tissue methods of grafting scion from the selected mature tree onto seedling root stock seems quite effective. Once the graft is successful and scion has grown the tip of the new shoot is grafted on another seedling. The procedure is repeated untill the scion exhibits juvenile characteristics and responds like a juvenile plant when placed in culture.

PLAN OF WORK

Basically the method proposed by Boxus (1974) for propagation of strawberry will be followed except the rejuvination technique. For that the method discovered by Franclet (1980) will be followed.

The plan of work will be divided into five phases.

FIRST PHASE

Sterilization of explant and culture of selected plant tip meristem.

SECOND PHASE

In vitro mass propagation of shoots.

THIRD PHASE

Grafting of propagules scions to seedling root stocks and rejuvination test.

FOURTH PHASE

Rooting of propagules.

FITH PHASE

Hardening of the plantlets.

A. STERILIZATION OF EXPLANT AND CULTURE OF TIP MERISTEMS

Apical or auxillary buds of orthotropic and plageotropic branches of field grown selected Pistacia cultivar will be the explant.

The treatment with different concentrations of disinfactants such as commercial bleach sodium or calcium hypochlorite, hydrogen per oxide, silver nitrate, or mercuric chloride will be given for explant sterilization.

B. IN VITRO MASS PROPAGATION OF SHOOTS

Contamination free explant will be transferred to the media containing one or more than one hormones suitable for shoot proliferation. Diallelic experiments (Anderson, 1980) will be followed while trying hormones in combination.

The environmental conditions such as temperature, intensity of light and photo period suitable for optimum growth will also be determined.

C. GRAFTING AND REJUVINATION TEST

i) Scion from *in vitro* proliferated shoots will be grafted on seedling root stock. Graftage will also be *in vitro*.

ii) Successfully grafted shoots will be again subjected to shoot proliferation.

iii) The buds from (ii) will be transferred to root forming media containing auxin. Appearance of roots at the basal ends of proliferated shoots or buds confirms rejuvination, otherwise the process is recycled i.e. scions (ii) are again grafted to fresh seedling root stocks, unless mature plant tissue rejuvinates.

Rejuvination of mature plant tissue will be determined by the number of shoot graftage and proliferation cycles.

D. ROOTING OF PROPAGULES

End product of C will be subjected to media containing one or more auxin in combination for rooting. The parameters will be same as described in B.

E. HARDENING OF PLANTLETS

Plantlets obtained from D will be provided with suitable environmental conditions such as light, temperature and humidity prior to transfer them in the field.

MATERIALS AND METHODS

MATERIAL:

Subject material will be seeds and buds of mature Pistachio cultivars.

METHOD:

1. ASEPTIC SEED GERMINATION

Method adapted by Barghchi and Alderson (1983) will be tried. Any modification suitable for aseptic growth of healthy aseptic seedling will also be made.

2. SHOOT MULTIPLICATION METHOD AND MAINTENANCE OF STOCK EXPLANT

Basically methods of Baraghci and Alderson (1985) will be followed for mature plant shoot tips, nodal buds or seedling shoots. If necessary modifications will also be adapted in due course.

3. GRAFTING TECHNIQUE

The technique of Optiz (1975, 77) and Navarro, Roistacher and Murashige (1975) described for pistachio graftage will be practiced.

4. ROOTING OF SHOOT TIPS

For rejuvenated shoot tips method of Barghci and Alderson (1983, 85) will be followed.

REFERENCES

Abbott, A.J. and Whitely, E. (1976)
Culture of Malus tissues in vitro. I Multiplication of apple plants from isolated shoot apieces.
Scientia Hort., 4, 183-189.

Anderson, W.C. (1980)
Tissue culture propagation of red raspberries. In proceedings of the conference on Nursery production of fruit plants through tissue culture - Applications and feasibility.
U.S. Dept. Agr., SEA, ARR-NE-11,
Beltsville Maryland, pp. 27-34.

Al-Barazi, Z. and Schwabe, W.W. (1985)
Studies on possible internal factors involved in determining case of rooting in cuttings of Pistacia vera and Prunus avium, CVS Colt and F 12/1.
J. of Hort. Sci. 60 (4), 439-445.

Al-Baraze, Z. and Schwabe, W.W. (1982)
Rooting soft wood cuttings of adult Pistacia vera.
J. Hort. Sci., 57, 247-252.

Baluch, B.A. (1979)
Pistachio cultivation
Ziraat Balochistan, 7 (1), 21-25. (Urdu)

Barghchi, M. and Alderson, P.G. (1985)
In vitro propagation of Pistachia vera L. and commercial cultivars Ohadi and Kalleghochi.
J. Hort. Sci., 60 (3), 423-430.

Barghchi, M. and Alderson, P.G. (1983)
In vitro propagation of Pistachia vera L. from seedling tissue.
J. Hort. Sci., 58, 435-445.

Boxus, P. and Quoirin, M. (1974)
La Culture de meristemes apicause de quelques especes de Prunus.
Bull. Sco. Roy. Bot. Belg., 107, 91-101.

Boxus, P., Quoirin, M. and Laine, J.M. (1977)
Large scale propagation of straw berry plants from tissue culture. In applied and fundamental aspects of plant cell, tissue and organ culture.
Reinert, J. and Bajaj, Y.P.S. eds. Springer - Verlag, Berlin, Heidelberg, New York, pp. 130-143.

Broome, O.C., and Zimmerman R.H. (1978)
In vitro propagation of black berry.
Hort Science, 13, 151-153

Driver, J.A., and Kuniyuki, A.H. (1984)
In vitro propagation of paradox walnut rootstock.
Hort Science, 19, 507-509.

Franclet, A. (1980)
Rajeunissement et propagation Vegetative Des Ligneux.
Ann. AFOCEL 1980., 12-41.

Harper, P.C. (1978)
Tissue culture propagation of black berry and tay berry.
Hort. Res., 18, 141-143.

Jones, O.P. and Hopgood, M.E. (1979)
The successful propagation in vitro of two root stocks of Prunus, The plum rootstock Pixy (P. insititia) and the cherry root stock F 12/1 (P. avium).
J. Hort. Sci., 54, 63-66.

Khan, Z.A. (1981)
Pistachio
Ziraat Baluchistan, 9 (3), 35-36. (Urdu)

Loreti, F., and Morini, S. (1982)
Mass propagation of fruit trees in Italy by tissue culture: Present status and perspectives.
Comb. Proc. Int. Plant Prop. Soc. 32, 283-291.

Morgan, T.H. (1901)
Regeneration, London : Macmillan.

Nasir, Y.J. (1983)
Anacardaceae
Fl. Pak., 152, 1-22.

Navarro, L.; Roistacher, C.N. and Murashige, T. (1975)
Improvement of shoot tip Grafting in vitro for virus - free citrus.
J. Amer. Soc. Hort. Sci., 100 (5), 471-479.

Optiz, K.W. (1977)
Training young Pistachio trees. University of California Division of Agricultural Sciences, Leaflet 2961, Berkeley.

Optiz, K.W. (1975)
The Pistachia nut.
University of California, Division of Agricultural Sciences, Leaflet No. 2279, Berkeley.

Schwann, Th. (1839)
Mikroskopische Untersuchungen uber die Uberreinstimmung in der struktur und dem Wachstume der Tiere und Pflanzen.
Leipzig: W. Englemann, Nr. 176, Oswalds Klassiker der exaktern Wissenschaften, 1910.

Zimmerman, R.H. (1985)
Application of tissue culture propagation to woody plants
In "Tissue culture in forestry and agriculture" Ed.
Henke, R.R.; Hughes, K.W.; Constantin, M.J. and Hollaender, A.
Plenum Publishing Corporation.

Zimmerman, R.H., (ed.) (1980)
Proceedings of the Conference on Nursery production of Fruit plants through Tissue Culture - Applications and Feasibility.
U.S. Dept. Agr., SEA, ARR-NE-11,
Beltsville, Maryland, 119 pp.

Zuccherelli, G. (1979)
Moltiplicazione in vitro dei portainnesti clonali del pesco.
Frutticoltura, 41 (2), 15-20.

Zucchereli, G.; Venturc, V. and Damino, C. (1978) Rapid propagation on a vast scale of Domasco 1869 rootstock by *in vitro* culture. *Ann. Ist. Sper. Frutt., Roma*, *9*, 21-23.

A STUDY OF THE CHEMICAL MANIPULATIONS INVOLVED IN THE CLONAL PROPAGATION OF PISTACIA VERA

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Title:- A STUDY OF THE CHEMICAL MANIPULATIONS INVOLVED IN THE CLONAL PROPAGATION OF PISTACIA VERA.

Subject:- SYNOPSIS FOR DOCTORAL WORK IN CHEMISTRY

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The global human population is continously expanding, and as a result concern is now being expressed in various quarters about the finite number of people world agriculture can support.

Plants enjoy their unique position as primary producers in all food chains, and as the only renewable energy source available to animals. The trapping of light energy from the sun and the production of organic molecules through photo synthesis, provides the basis of not only all food on earth, but also all life on earth. Plants directly supply 90 % of human calorie intake and 80 % of the protein intake, the remainder being derived from animal products although these animals have ultimately derived their nutrition from plants. The vast majority of this food, 90 %, is produced on land.

Man has so far used approximately three thousand plant species as food, and around 20 crop species furnish majority of the protein and calories requirements these days.

Conventional types of plant breeding have had remarkable success this century in improving the yield and quality traits of a wide range of plants. About half of the increases in yields can be attributed to positive breeding and selection of lines that are resistant to

pests and diseases and that are also tolerant to many stressful environmental conditions. The remainder of these increases is attributed to general improvements in agronomic practices and more wide spread use of sophisticated agro chemicals[1].

Although the use of fertilizers have increased the crop yield, and propagation rates have been enhanced, this method still has some of the limitations of its own. The time taken to produce and test the plant varieties is an important limiting consideration and the germination through seeds is also prone to genetic variations.

With better understanding of the biochemistry and physiology of plants the scientists have now been able to skilfully manipulate the chemical requirements of plants *in vitro*. which has resulted in the emergence of cell suspension culture technique.

Cell culture technique was first reported by Muir[2], who found that callus fragments when transferred to a liquid medium and agitated on a shaker, gave a cellular suspension that could be propagated by sub-culture. Pioneering works in the field of cell suspension culture have been reported by scientists like Street[3], Steward[4], Lamport[5], Gamborg[6], Miller[7] etc.

Cell culture technique has provided insights into many areas including totipotency, differentiation, cell division, cell nutrition, metabolism, radio-biology, and cell preservation. Production of haploid plants and the induction of somatic hybridization are the great benefits that have been derived out of the recent developments in cell culture technique. Similarly cell culture technique is proving to be an efficient method for the commercial production of natural products.

Cell culture technique is now being extensively used for the enhanced propagation of many plant species. A large number of plants can be produced from a given amount of explant material within a short period of time when adventitious shoots or embryos are induced in cell suspension cultures. If a large proportion of the cultured cell population is made up of totipotent cells of cell clusters, extremely high number (10^5 - 10^6) of plants can be produced from a few 100 ml cultures within a single culture generation[1].

During incubation the amount of cell material increases; this increase occurs only for a limited time and the culture reaches a point of maximum yield of cell mass. The cell culture is then subcultured, and then in a subsequent and

similar incubation period it goes through a similar pattern of growth and yield a similar amount of cell material. Thus the culture can be continously propagated by successive batch cultures of appropriate duration and the number of stock cultures increased at regular intervals[8].

It has been demonstrated on both the laboratory and commercial scales that the mass propagation of ornamental species such as lily and vegetables like celery and carrot is possible in liquid culture. Clonal propagation capability has been demonstrated for some woody plants like coconut, oil palm and date palm etc. Mass production of elite adult trees is expected to produce dramatic improvements in forest productivity.

Agro chemistry has developed steadily over the years. As a result scientists have now been able to have a better understanding of the chemical requirement of plants *in vivo* and *in vitro*. There are 18 indispensable elements, which are required by the plant for its growth *in vivo* and *in vitro*. These include, carbon, oxygen, hydrogen, nitrogen, phosphorous, potassium, calcium, magnesium, sulphur, boron, copper, iron, zinc, mangnese, molybedinum, cobalt, sodium and chlorine. These elements perform

important function in the plants. Some have important role in osmotic phenomena, some are fundamental constituents of metabolic products and others may act catalytically in the regulation of various chemical processes within the cell.

Carbon, hydrogen and oxygen are nutritive elements. They form cellulose, lignin, reserve food and most of the protoplasm of plants. Nitrogen is one of the constituent of proteins, which form major part of protoplasma. It is received from the soil through nitrate, nitrite and ammonium salts. Phosphorous is taken up by the plant as ions of PO_4^{3-}, H_2PO_4 and HPO_4^{2-}. It is an essential part of nucleo-proteins. It is also necessary for the formation of nucleic acids and lecithin. It plays important role in transormation of carbohydrates and respiration. Potassium is believed to be related to the formation of starch and sugar. It is essential for the enzymatic hydrolysis of starch. Potassium helps iron in chlorophyll formation. Calcium enters into the constitution of cell wall, especially middle lamella, in the form of calcium pectate. It regulates the uptake of other ions from the soil. Ions of calcium antagonize the absorption of potassium ions by roots. Magnesium is

a constituent of chlorophyll and is, therefore, indispensable to green plants. It is necessary for the formation of nucleo-proteins and it probably has a part in respiratory enzyme reactions. Sulphur is taken up by the plant in the form of SO_4^{--} and it enters into the chemical composition of proteins.

The micronutrients required are Fe, Bo, Cu, Zn, Mn and Mo. Iron is necessary for chlorophyll formation as it may be acting as a catalyst. It is also concerned with respiration. The specific action of boron is not known, but there is evidence that it is concerned with respiration. Boron promotes the absorption of calcium. Copper is concerned with respiration and influences the use of iron. Manganese is necessary for enzymes related to dehydration and decarboxylation. Similarly molybdenum is essential for nitrogen metabolism, nitrogen fixation, and for the production of ascorbic acid.

On the basis of these and other informations, various research workers have formulated different mediums, with appropriate combinations of inorganic and organic compounds, for <u>in vitro</u> growth of cell cultures. These include Murashige and Skoog's medium[11], White's medium[9], Gamborg's medium[6], Schenk and Hildebrandt's medium[10], Anderson's medium[12], Reynold and Murashige's medium[13] etc.

The composition of the culture medium is an important factor in the successful establishment of a cell culture. The nutritional requirements of plants varies not only from plant to plant, but even from one part to the other, within the same plant, so the success with the cell culture can be achieved only by the use of suitable nutrient media and by providing necessary chemicals in appropriate combination and forms. The ingredients of various plant culture medias may be conveniently categorised as follows:-

MACRO NUTRIENTS:

Sulphates, chlorides, nitrites and phosphates of N, K, P, Ca, S and Mg in millimole quantities.

MICRO NUTRIENTS:

Micro molar concentrations of micronutrients are furnished by sulphates of Ni, Fe, Na, Mn, Zn, Cu, Be and Ti, and chlorides of Ni, Co, Al and Fe, lodide of K and tartarate of Fe also provide the micromolar concentration of K and Fe.

CARBOHYDRATES:

Carbohydrates most commonly used are sucrose and D-glucose. Other carbohydrates that may be used include D-fructose, Lactose, Maltose, Galactose and Starch.

41

VITAMINS:

Thiamine-HCl is the most commonly used vitamin. Other vitamins that have been reported to have been used are nicotinic acid (niacin), pyridoxine (vitamin B_6), P-amino-benzoic acid (PABA; vitamin B_x), ascorbic acid (vitamin C), biotin (vitamin H), choline chloride, cyanocobalamin (vitamin B_{12}), folic acid (vitamin B_c), calcium pantothenate, and riboflavin (vitamin B_2).

AMINO ACIDS AND AMIDES:

Most extensively used amino acid is glycine (amino acetic acid) Casein hydrolysate and casamino acids are also extensively used. Other amino acids and amides commonly used are L-aspartic acid, L-asparagine, L-glutamic acid, L-glutamine, L-arginine and L-methionine.

COMPLEX NATURAL SUBSTANCES:

Some of the commonly used complex natural substances are banana pulp, coconut endosperm, fish emulsion, malt extract, orange juice, protein hydrolysates, tomato juice, and yeast extract.

PLANT GROWTH REGULATORS:

Auxins, cytokinins, gibberellines and morphactins constitute the major groups of plant growth regulators.

Auxins play their role in root promotion. Commonly used auxins are 2,4-dichloro phenoxyacetic acid (2,4-D), Indole -3-acetic acid (IAA), α-napthalene acetic acid (NAA), Indole -3-butyric acid (IBA), 2,4,5-trichloro-phenoxy acetic acid (2,4,5-T), p-chloro-phenoxy acetic acid (4-CPA), Indole -3-valeric acid (IVA) etc.

Cytokinins find their role in shoot promotion and commonly used cytokinins are benzyl adenine, diphenyl urea, kinetin, zeatin, IPA, etc. Morphactins are used as the stimulant for lateral bud growth. Similarly gibberallinencs are used to promote flowering.

It may therefore, be concluded that the cells originating from the plant kingdom may be grown in vitro in the mediums consisting of appropriate combination and concentrations of the organic and inorganic compounds mentioned above.

An important element in scientific progress is the development of new techniques which either enable more critical experiments to be undertaken or which render certain problems accessible to experimental study for the first time. Cell culture technique must therefore be evaluated in terms of the scientific progress it has made or will make possible in the future. Cell culture technique

which was initially employed for the propagation of herbacious plants has open many facets of cell-biochemistry, biochemical genetics and plant breeding from both fundamental and practical stand points.

Some of the herbacious plants that have been successfully propagated by cell culture technique include:-

Lycopersicum esculentum	Nover *et al.*[14],
Catheranthus roseus	Bailey *et al.*[15],
Rice	Zhou *et al.*[16],
Sugar-cane	Komer *et al.*[17],
Red clover	Philips *et al.*[18],
Alfalfa	Stuart *et al.*[19],
Solanum melongena	Gleddie *et al.*[20],
Solanum lycopersicoides	Handley *et al.*[21],

In recent years, methods of cell culture that provide rapid vegetative propagation of herbacious plants have been applied successfully to woody species. Initially these methods met with little success with trees, and upto five years ago the majority of reports on such propagation *in vitro* revealed only low or sporadic production of woody plants. There was a dire need for the developments, so

that the cell culture technique may be effectively employed on woody plants, as they have to face more problems as compared to the herbacious plants. They have a longer life cycle, which means a longer regeneration time, and as a result mutation and adaptation rate is very slow. Thus if a degenerative evolutionary trait becomes dominant, then it becomes very difficult to protect that plant specie against degeneration. Some other problems which are encountered by woody plants include very late determination of sex, improper means of propagation, sterility etc. But the recent progress has been excellent and already the success of *in vitro* propagation with adult trees is bringing important practical benefits and so the future of propagation *in vitro* as an invaluable aid to the cropping of trees appears to be assured.

The woody species that have been successfully propagated in recent years include.

Phellodendron amurensis	Yamada *et al.*[22]
Pseudotsuga menziesii	Kirby[23]
Citrus sp.	Ben-Hayyim *et al.*[24]
Pinus contorta	Webb[25]
Musa "ABB" c/v *Saba* and *Pelipita*	Cronaver *et al.*[26]

Pimpinella anisum L.	Ernst et al.[27]
Populus tremuloides	Noh, Eun woon et al.[28]

It will therefore be useful to undertake a study for designing a model for hastening the regenerative process of a woody plant such as Pistacia vera. Pistacia vera is an inhabitant of Baluchistan, and is an important cash crop. Cultivators are facing a loss of time and labour as pistachio plantation is suffering from a number of problems such as undesirable male to female ratio, very late determination of sex, inadequate nursery stock, and the long time 8-15 years often required for an orchad to come into commercial production. As the work on the propagation of Pistacia vera through tissue culture, is at present being undertaken in the tissue culture laboratory of P.C.S.I.R. Laboratories, and some studies on regeneration through callus cultures has been successful, thus the aim of the present study is to develop a procedure and formulation of medium for in vitro growth and development of Pistacia vera through cell suspension culture.

"AIMS AND OBJECTIVES"

1. Main object of the study is to develop a procedure for the clonal propagation of healthy desired pistachio cultivars.

2. It is aimed to apply cell culture technique to achieve this object.

PLAN OF WORK

Studies will be carried out to formulate chemical compositions of macro nutrients, micro nutrients, organic supplements and hormones for a medium suitable for the growth and maintenance of cells derived through callus as obtained from tissue explant from local Pistachio cultivars. Best possible changes will be incorporated to improve the medium composition.

The plan of work is divided into three stages.

STAGE NO. I

INDUCTION OF CALLOGENESIS

Explant material will be cultured on media for the induction of callogenesis. Different salt mixtures and concentration and combinations of cytokinins and auxins will be tried to initiate callus formation. Some of the complex natural supplements which are supposed to be helpful in callus initiation will also be tried.

Since major work is related with cell culture and a highly friable callus is desired, so special studies will be undertaken for not only the initiation but also

for an induction of a high degree of friability in the callus. The callus obtained in this way may subsequently produce a high degree of friability in the suspension.

Guidance will be sought from the work done by Gamborg[6], Murashige and Skoog[11] etc.

STAGE NO. II

"INITIATION OF SUSPENSION CULTURE"

Trials will be made for the selection of chemical constituents and composition of nutrient media for initiation of suspension culture.

Work of stage No. II will comprise of the following studies.

II (a) DETERMINATION OF MEDIA

Different medium formulations for an effective increase of biomass will be tried. Determination of quantities of growth regulators and their combinations will form as integral part of the study.

II (b) DETERMINATION OF MEDIA CONDITIONS

Special considerations will be given to the studies leading to appropriate selection of pH, light, humidity and temperature. As pH plays a very important role in suspension culture, so as to stabilize the pH different substances like EDTA, nitrogen, calcium dihydrogen orthophosphate, calcium phosphate and calcium carbonate will be tried.

II (c) INDUCTION OF FRIABILITY

As it is desired to have a suspension culture consisting of high degree of friability, so certain factors like, wall degrading enzymes and increasing the auxin and decreasing the cytokinin concentrations will be tried. Guidance will be sought from the work of Reinert and White[29], Koji Nomura and Atsushi Komamine[30], Venketes Waran[31], Walter Tulecke[40] etc.

STAGE NO. III

INDUCTION OF EMBRYOGENESIS AND ORGANOGENESIS

Once the biomass of required density has been obtained, steps would be taken to induce organogenesis and embryogenesis. The work of stage No. III will be divided as such.

III (a) INDUCTION OF RHIZOGENESIS

Determination of hormonal quantity and their combination and medium formulations for the induction of rhizogenesis.

III (b) INDUCTION OF CAULOGENESIS

Determination of hormonal quantity and their combination and medium formulations for the induction of caulogenesis.

III (c) INDUCTION OF EMBRYOGENESIS

Determination of hormonal quantity and their combination and medium formulations for the induction of embryogenesis.

Work done by Reinert[41], Butenko[32], Steward and Mapes[33], Fujimura, T. and A. Komamine[34], Street[35], Ludwig[36] etc. will provide the required guidance.

MATERIALS AND METHODS

1. Seeds of local Pistachio cultivars will be used as the source of subject material.

2. CULTURE TECHNIQUE

Stationary culture technique will be applied throughout the study for obtaining callogenesis. Filter paper bridge may also be applied.

3. INGREDIENTS OF MEDIA

As stated above several media have been developed by various workers to suit particular requirements of a cultured tissue and cells. Consequently, several standard media will have to be tried to find out the best suitable one. Many modifications in the concentration and forms of chemical constituents of culture media will be tried to formulate the best chemical composition of media for Pistachio explant in both tissue and cell culture. The ingredients of the medium will be as following;-

a) SALT MIXTURES

Salt mixtures of MS, B5, SH, RM or WPM and their modifications.

b) ORGANIC COMPOUNDS

Carbohydrates, vitamins, amino acids and hormonal substances such as auxins and cytokinins.

c) NATURAL PRODUCTS

Complex natural preparations such as coconut milk, orange juice, tomato juice, malt extract or yeast extract will be used if any need arises to modify the medium composition.

4. PREPARATION OF CULTURE MEDIA

a) Tissue culture media will be prepared by the standard methods as formulated and described by Gamborg and Shyluk[37], and Tasaki[38].

b) Cell culture media will be prepared by the standard methods as formulated and described by various workers such as Gamborg and Skyluk[37], Tasaki[38] and Huang and Murashige[39].

5. QUANTITATIVE ESTIMATION

Quantitative estimation of cell suspension culture will be undertaken by any one of the following methods.

a) Determination of mitotic index.

b) Estimation through cell counting.

All the equipments available in tissue culture laboratory over here in PCSIR Laboratories and if required, the equipments in Punjab University will be utilized.

REFERENCES

1. Mantell, S.H.; Mathews, J.A.; and Mckee, R.A., (eds), (1985), in "Principles of plant biotechnology", pp 1-8. Blackwell scientific publications, Oxford.

2. Muir, W.H.; Hilderbrandt, A.C.; and Riker, A.J. (1954). Plant tissue cultures produced from single isolated plant cells. Science, N.Y., 119, 877-887

3. Street, H.E.; Collins, H.A.; Short, K.C.; Short, K.C.; and Simpkins, I. (1968). Hormonal control of cell division and expansion in suspension cultures of Acer Pseudoplatanus L.: the action of kinetin, pp. 489-504, in "Biochemistry and physiology of plant growth substances", F. Wightman and G. Setter field, (eds) Runge press, Ottawa.

4. Steward, F.C.; Caplin S.M.; and Millar, F.K. (1952). New techniques for the investigation of metabolism, nutrion and growth in undifferentiated cells. Ann. Bot., 16, 58-77

5. Lamport, D.T.A. (1964). Cell suspension cultures of higher plants : isolation and growth energetics. Exp. Cell Res., 33, 195-206

61

6. Gamborg, O.L.; Miller, R.A.; and Ojima, K. (1968).
Nutrient requirements of suspension cultures of soybean root cells.
Exp. Cell Res., 50, 151-158

7. Miller, C.O.; Skoog, F.; Okumura, F.S.; and Strong, F.M. (1956).
Isolation, structure and synthesis of kinetin, a substance promoting cell division.
J. Am. Chem. Soc., 78, 1375-1380

8. Street, H.E.
Cell (suspension) cultures - techniques.
pp 61-62 in "Plant tissue and cell culture", ed. Street, H.E. Blackwell scientific publications, Oxford.

9. White, P.R. (1963).
White's standard nutrient solution.
Ann. Bot., 47, 133-139.

10. Schenk, R.V.; and Hildebrandt, A.C. (1972).
Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures.
Can. J. Bot., 50, 199-204

11. Murashige, T.; and Skoog, F. (1962).
A revised medium for rapid growth and bioassays with tobacco tissue cultures.
Physiol. Plant, 15, 473-97

62

12. Anderson, W.C. (1978).
Tissue culture propagation of rhododendrons.
In vitro, 14, 333

13. Reynolds, T.F. and Murashige, T. (1979).
A sexual embryogenesis in callus cultures of palms.
In vitro, 15 (5), 383-387

14. Nover, L.; Kranz, E.; Scharf, K.D. (1982).
Biochem. Physiol. Pflanz., 177 (6), 483-489

15. Bailey, C.; Nicholson, H.; Morris, P.; Smart, N.J. (1985).
A simple model of growth and product formation in cell suspensions of Catharanthus roseus.
Appl. Biochem. Biotechnol. 11 (3), 207-19

16. Zhou, Gaozhi; Ye, Mingming; Ge, Koulin; Yang, Jinshui; Wang, Yunzhu. (19[illegible]).
Rice single cell cultivation [illegible] plantlet regeneration.
Fudan Xuebao, Ziran Kexueban 25 (3), 335-40 (ch.)

17. Komor, Ewald; Thom, Margaret; Maretzki, Andrew. (1981).
Charge balance and acidity regulation during growth of sugar-cane cell suspension.
Plant Cell environ., 4 (5), 359-365

18. Philips, Gregory, C.; Colins, G.B. (1980).
Somatic embryogenesis from cell suspension cultures of red clover.
Crop Sci., 20 (3), 323-326

19. Stuart, David A.; Nelson, Janet; Strick land, Steven G.; Nichol, James, W. (1985).
Factors affecting developmental processes in alfalfa cell culture.
Basic. life Science, 32, 59-73

20. Gleddie, S.; Keller, W.A.; Setherfield, G. (1986).
Somatic embryogenesis and plant regeneration from cell suspension of Salanum melongena.
Can. J. Bot., 64 (2), 355-361

21. Handley, Levis W.; Sink, Kenneth C. (1985).
Plant regeneration of protoplasts isolated from suspension cultures of Solanum lycopersicoides.
Plant Sci. (Limerick, Irel), 42 (3), 201-207

22. Yamada, Y.; Kuboi, T.; Sato, F. (1981).
Cell differentiation.
Plant tissue culture (Proc. Symp.) 1978.
371-389

23. Kirby, Edward G. (1982).
The effects of organic nitrogen sources on growth of cell cultures of Douglas-fir.
Physiol. Plant, 56 (1), 114-117

24. Ben-Hayyim, Gozal; Kochba J. (1982).
Growth characteristics and stability of tolerance of citrus callus cells subjected to NaCl stress.
Plant Sci. Lett., 27 (1), 87-94

25. Webb, K. Judith.
Growth and cyto differentiation in cell suspension cultures of Pinus contorta.
"Coll oq. Int. Cult, "in vitro" Essence for., Expo 1981, 217-226

26. Cronaver, S.; Korikorian, A.D. (1983).
Somatic embryos from cultured tissues of triploid plantains (Musa "ABA").
Plant Cell. Rep., 2 (6), 289-91

27. Ernst, D.; Oesterhelt, D.; Schaefer, W. (1984).
Endogenous cytokinins during embryogenesis in an anise cell culture (Pimpinella anisum).
Planta, 161 (3), 240-245

28. Noh, Eun Woon; Minocha, Subhash C. (1986).
High efficiency shoot regeneration from callus of Populus tremuloides.
Plant Cell Rep., 5 (6), 464-467

29. Reinert, J., and White, P.R. (1956).
The cultivation in vitro of tumor tissues and normal tissues of Picea glauca.
Physiol. Plant, 9, 177-189

30. Koji Nomura and Atsushi Komamine (1985). Identification and isolation of single cells that produce somatic embryos at a high frequency in a carrot suspension culture. Plant Physiol., 79, 988-991

31. Venkateswaran, S.; and Peter, K. Chen. Nutritional factors affecting growth, friability, and lignification of suspension cultures of higher plant cells. Can. J. Botany, 42, 1279-1286

32. Butenko, R.; Bychkova, G., Zholkerich, V. and Perisakhzon, B. (1974). Synchronization of cell division and some peculiarities in the mitotic cycle in suspension cultures of Panax gin seng. Proceedings of the 3rd. International Congress of plant tissue and cell culture, University of Leicester, Leicester.

33. Steward, F.C., Mapes, M.O., and Smith, J. (1958). Growth and organized development of cultured cells. I. Growth and division of freely suspended cells. Am. J. Bot., 45, 693

34. Fujimura, T. and Komamine, A. (1980). The serial observation of embryogenesis in a carrot cell suspension culture. New Phytol., 86, 213-218

35. Street, H.E., and G.G. Henshaw (1963).
Cell division and differentiation in suspension cultures of higher plant cells.
Symp. Soc. Exptl. Biol., 17, 234-256

36. Ludwig Bergmann (1960).
Growth and division of single cells of higher plants in vitro.
The Journal of General Physiology, 43, 841-851

37. Gamborg, O.L. and Shyluk, J.P. (1981).
Nutrition, media and characteristics of plant cell and tissue cultures, pp 21-44, in "Plant tissue culture", Trever A. Thorpe, (ed) Academic Press, New York.

38. Tasaki, S. (1985).
Experiment on tissue culture.
Vegetable course, TIATC, pp 1-12.

39. Huang, Li-chan and Murashige, T.
Plant tissue culture media: Major constituents their preparation and some applications.
TCA Manmeal, 3 (1), 539-548

40. Walter Tulecke, Ralph Taggart, and Luke Colavito (1965).
Continuous cultures of higher plant cells in liquid media.
Copy right, 1965 by Boyce Thompson institute for plant research, 23, 33-46.

41. Reinert, J. (1963).
Growth of single cells from higher plants on synthetic media.
Nature, Lond., 200; 90-91

61

EFFECT OF ORGANIC AND INORGANIC NUTRIENTS AND HORMONES ON GROWTH AND DEVELOPMENT OF TISSUE EXPLANT FROM LOCAL PISTACHIO CULTIVARS CULTURED IN VITRO

A SYNOPSIS SUBMITTED FOR DOCTORIAL WORK IN CHEMISTRY

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EFFECT OF ORGANIC AND INORGANIC NUTRIENTS AND HORMONES ON GROWTH AND DEVELOPMENT OF TISSUE EXPLANT FROM LOCAL PISTACHIO CULTIVARS CULTURED IN VITRO

INTRODUCTION

The world population is expanding logrithmically. It is estimated that it will be 6 billion by the turn of the century. This would mean expansion in demand for food which would necessitate affective utilization of the methods for increasing the production of food. The quantity of food can be increased by increasing the yield per hectare of the crops. This goal can be achieved by using better yielding seeds or by increasing inputs or by improvement in the cropping patterns.

Agriculture sciences have been gradually developed to cope with such problems, the traditional means are replaced by more sofisticated techniques which can be more promising and fruitful. Plant tissue culture technology is one such tool which can be used for studying the basic and applied problems of food producing crops. This technology holds out the promise of high yielding hybrids and disease resistant strains which can result in the rapid propagation of plants. Thus it will ensure an increase in the production of such commodities.

The underlying principles involved in plant tissue culture are very simple. Firstly, it is necessary to isolate a plant part from its inter-organ, inter-tissue or inter-cellular relationship with the intact plant. Secondly it is important to provide that plant part with an appropriate environment in which it can express its internsic or induced potential. The culture environment includes the physical conditions (temperature, humidity, light intensity and photoperiod etc.), and chemical composition of the medium - which is a mixture of organic and inorganic components taken in specific micro and macro quantities.

Physical conditions can only be effective if the compositic of the culture medium is suitable for the plant part in culture. The requirement of the plants varies not only from plant to plant but even from one part to the other within the same plant. It means that the chemical composition of the culture medium is of primary importance. So it is concluded that success with the plant and organ cultures can be achieved by the use of suitable nutrient media by providing necessary chemicals in appropriate combinations and forms. A lot of work has been done to formulate variety of media suitable for different plants.

As reported in the literature the ingredients of the plant tissue culture media can be categorized as follows:-

a) Inorganic Salt Mixture:

Efforts to optimize the needs of specific plants have resulted in several salt mixtures. These mixtures contain varying quantities of macronutrients (N, Mg, P, K, Ca and Cl) and certain micronutrients including traces of Cu, Zn Mn, Fe, B and Mo. These salt mixtures are named after the scientist's name who have formulated that salt mixture such as MS medium by T. Murashige and F. Skoog (1962). White's medium by P.R. White (1963) and RM Medium by J. Reynolds and T. Murashige (1979), etc.

b) Organic Compounds:

The critical category of organic compounds includes, carbohydrates, hormonal substances and vitamins. Benefits can often be obtained from supplements of amino acids and/or amides, certain purines and/or pyrimidines, hexitoles and organic acids. 2 - 3 % sucorse is the most extensively employed carbon source. Higher concentrations 5 - 12 % may also be advantageous for some species or for some plants (Hildbrandt and Riker, 1953; Nickell and Maretzki, 1970).

Auxins and Cytokinins are the growth regulators. Auxins are the root promoting and the cytokinins are shoot promoting hormones. Different plant parts response to different combination and concentrations of these hormones have to be traced out for organogenesis/regenration studies. They are effective in very low concentrations as according to Huang and Murashige (1976) i.e. auxin concentration should not exceed the range of 0.1 - 10 mg/L whereas the concentration of cytokinins can range from 0.03 - 30 mg/L. The only vitamin which has shown consistent importance in plant tissue culture is thiamine-HCl (vitamin B_1) whereas nicotinic acid (niacin) and pycidoxin-HCl (vitamin - B_6) may also be added.

To fulfil the requirements of any explant cultured in vitro, it is necessary to formulate a medium to provide it with best cultural conditions. Many plants have been regenerated through callus formation by tissue culture for example date palm, grapes, potato etc. yet there is a large number of species which are left neglected, though they need tissue culture application due to certain problems they are facing. Pistachio nut is one of them. It is a woody plant therefore its tissue culture is quite difficult.

Tissue culture or aseptic culture of woody plants, although, introduced in the early thirties, is still considered to be a new field in several countries of the world including Pakistan.

One of the reasons is that, most of the research done was basic and results were confined to the four walls of the laboratories. Further more a small number of scientists were in this field.

During the last three decades, there has been a growing awareness about the economic impact and application which plant tissue culture techniques can have in many important fields, such as agriculture, horticulture, arboriculture and ornamental. The relem of tissue culture also covers the production of medicinal and enzymatically important substances. In fact crop improvement is the most vital and rewarding area of research in agriculture and horticulture especially.

Pakistan being an agricultural country needs improvement in all these areas. So it is the need of time to adapt tissue culture technology to work for the plant species endangered or facing certain problems related to genetics or means of propagation in our country. Pistachio nut is one of those species - a dicotyledonous dioceous plant (separate male and female plants) inhibitant of Baluchistan area.

Pistachio nut is an important cash crop, arborists of Baluchistan are facing loss of time and labour due to slow rate of propagation and lack of sex determination at early stages of plantation. As mentioned before it suffers from improper means of propagation, aflatoxin contamination inappropriate male to female plant ratio& delayed expression of sex. Pistachio is either

propagated by seeds or by budding. But there are problems related to the formation of satisfactory bud union. Seed germination is faced by two major draw backs; first is the male to female ratio and the second one is the genetic variations which is undesirable. Unfortunately the female to male ratio in Pakistan has been observed to be 1 : 10 - 12. Sex of the plant is determined on flowering after 7 - 8 years of plantation, which is very uneconomical. Pistachio nut trees are also prone to drought and fungal attacks. Drought could be fatal and pathogenic attack could result in contamination of the nuts with aflatoxin. Due to these problems it will be useful to undertake a study for designing a model for hastening the regenerative process of Pistachio nut in Pakistan as well as in other countries. Because of the successful application of the tissue culture techniques for the woody dicotyledonous plants is reported to check the degenerative process.

Tissue culture of trees has been reviewed several times recently (Bonga and D-urzan 1977; Sommer & Linda 1981). Since woody tree tissue culture is still mostly at the experimental stage more research is required to adapt it to large scale for industrial use (Bonga & D-urzan 1982). The first report of organogenesis in tree tissue culture was made by Gautheret (1940). In vitro shoot regeneration of fruit bearing trees such as Quandong and Plum bush (Santalum lenceolatum) have been reported by Barlas et al., (1980). It is reported in literature,

(Rao & Bapat 1978) that the establishment of successful procedures for deriving a large number of plants through the regeneration of shoot buds in culture of hypocotyl segments. Santalam album also showed a high potential for the regeneration of multiple shoot buds on a basal medium supplemented with benzyladenine BA, 1 mg/L (Bapat & Rao, 1979).

It is also reported that tissue culture technology has been employed to major fruit and nut crops, which are vegetatively propagatable in vitro as listed below:

Aclinidia chenensis Harada (1975)	
Anaas comosus L.	Merru. Pannetier & Lonand (1976)
Carica papaya L	Yie & Liaw (1977)
Citrus sp.	Chaturvedi & Mitra (1974)
Coffea arbica L.	Sondahl & Sharp (1977)
Malus sylveslsis Mill	Jones et al., (1977)
Fragaria cheloensis Duchesne	Boxus et al., (1977)
Diospyros kaki L.f.	Yokoyama and Takeuchi (1976)
Corylus auellama L.	Radojevic et al., (1975)

Due to highly heterozygous nature of pistachio trees and to the difficulties encountered with the rooting of shoot cuttings, commercial cultivars are propagated by comparatively slow and expensive methods of grafting or buding scion on to the seedling's root stock (Joley, 1969; Whitehouse, 1957, Optiz, 1975, 1976, Maggs, 1982). As mentioned before drawback to this method is the

failure to the formation of satisfactory bud union. Therefore the development of rapid means of clonal propagation of elite Pistachio varieties is of prime importance. It is reported that clonal propagation of Pistacia vera has been achieved through multiple shoot proliferation and there rooting. Al Barazi & Shwabe (1982) observed that Indol butaric acid is suitable for the rooting of soft wood shoots of pistachio. Barghchi & Alderson (1983;1985) both formulated nutrient media for clonal propagation of pistachio using explants of different age. In 1983 they reported propagation through seedling material while in 1985 they formulated a medium for multiplication of shoots and their rooting from explant of 1 - 2 years old plant.

As mentioned above multiplication of shoot and their rooting has been achieved but propagation through callus formation which can expedite the multiplication rate is still not reported. Thus the aim of the present study is to develop a procedure and formulation of medium for in vitro growth and development of tissue from Pistachio through tissue culture. An experimental scheme proposed by Murashige (1977) will be helpful to identify the parameters that require systematic evaluation to develop a tissue culture procedure for the propagation of Pistachio.

AIMS AND OBJECTS:

1) Main object of the study is to develop a procedure for the clonal propagation of healthy desired Pistachio cultivars.

2) It is aimed to apply tissue culture technology to achieve the object. For this purpose chemical formulation of culture media suitable for the growth and development of sterile explant from local pistachio cultivars will be explored.

PLAN OF WORK:

Studies will be carried out to formulate chemical compositions of macro nutrients, micro nutrients, organic supplements and hormones for a medium suitable for the growth and maintenance of tissue explant from local pistachio cultivars. Best possible changes will be incorporated to improve the medium composition. Plan of work will be divided into two stages:-

STAGE I:

Effect of Physical and Chemical Factors on Callogenesis:

Determination of chemical nutrients, their composition and concentrations suitable for callogenesis in pistachio explant.

I a: In order to select disinfectant and to maintain conditions for seed sterilization, different sterilizing agents will be tried. Calcium and sodium hypochlorite, hydrogen peroxide, silver nitrate, mercuric chloride or certain antibiotics will be individually employed. Determination of proper sterilizing agent, its effective concentration and soaking time period leading to aseptic germination of seeds will provide the subject material.

I b: Determination of Callogenesis inducing factors:

Sterile explant material from aseptically germinated seedlings will be cultured on media for the induction of callogenesis. Different concentrations and combinations of

cytokinins and auxins will be tried to initiate callus formation. Some of the complex natural supplements which are supposed to be helpful in callus initiation will also be tried. The chemicals are specified in materials and methods.

STAGE II:

Effect of Physical and Chemical Factors on Organogenesis:

Trails will be made for selection of chemical constituents and composition of nutrient media for induction of organogenesis. A general guidline prescribed by Murashige (1977) for manipulation of organ initiation will be helpful for organogenesis in pistachio.

II a: Determination of harmonal quantities and their combination as well as medium formulation for rhizogenesis will be tried.

II b: A parallel procedure will be adapted for shoot initiation.

II c: Portions of callus will be subjected to medium without hormones for embryo formation.

MATERIALS AND METHODS

1. Sterilization and Germination of Seeds:

   a) Seeds of local pistachio cultivars will be used as the source of sterile plant material.

   b) Method of seed selection and sterilization described by Gamborg and Shyluk (1981) will be followed.

2. Culture Technique:

   Stationary culture technique will be applied throughout the study, occassionally paper bridge technique will also be tried.

3. Ingredients of Media:

   Medium ingredients will include:-

   a) Salt mixture - MS, SH, B_5, RM etc. and their modifications.

   b) Organic Compounds: Carbohydrates, vitamins, amino-acids and harmonal substances such as auxins and cytokinins.

   c) Complex natural preparations such as coconut milk orange juice, tomato juice, etc.

4. Preparation of semi-solid Culture Media:

a) Tissue culture media will be prepared by the standard methods as formulated and discribed by various workers such as Murashige & Skoog (1962); Gambarg (1968); Schenk & Hildbrandt (1972) and Reynolds & Murashige (1979).

b) General instructions provided by Huang & Murashige (1976) for the preparation of major constituents including macro and micro nutrients, organic and natural supplements of plant tissue culture media will be followed.

5. Selection of Culture Media:

Typically in attempting to establish a new species *in vitro* culture a screening system is used. Explants are tested on several media to find out the best media composition for the explant under observation. (Reinert & Bajaj, 1977). As stated before several media have been developed by various works to suit particular requirements of a cultured tissue. Consequently, several standard media will have to be tried to find out the best suitable one. Many modifications in the concentrations and forms of chemical constituents of the culture media will be tried to formulate the best chemical composition of media for pistachio explant in culture.

Facilities available

1. Strile incubation chamber
2. pH meter
3. Incubation rooms.
4. Moisture determination balance
5. Shakers
6. Spectrophoto meter
7. Microtome
8. Autoclave
9. Microscopes (disecting, research compound)

All other equipments in the tissue culture laboratory in Food Technology and Fermentation Division, PCSIR Laboratories, Lahore and Department of Chemistry, Punjab University will be available to use according to the requirement of the research work.

REFERENCES

1. Al Barazi, Z. and Schwabe, W.W. (1982).
Rooting softwood cuttings of adult Pistacia vera
J. Hot. Sci., 57, 247-252

2. Barghchi, M. and Alderson, P.G. (1983).
In vitro propagation of Pistacia vera L. from seedling tissue.
J. Hort. Sci., 58, 435-445

3. Barghchi, M. and Alderson, P.G. (1985).
In vitro propagation of Pistacia vera L. and commercial cultivars Ohadi and Kalleghochi.
J. Hort. Sci., 60, (3), 423-430

4. Barlass, M.; Grant, W.J.R. and Skene, K.G.M. (1980).
Shoot regeneration from native Australian fruit bearing trees - Quandong and Plumbush.
Aust. J. Bot., 28, 405-409

5. Bapat, V.A. and Rao, P.S. (1979).
Somatic embryogenesis and plantlet formation in tissue culture of Sandal wood (Santalum album L.).
Ann. Bot., 44, 629-630

6. Bonga, J.M. and Durzan, D.J. (1977).
In: "Applied and fundamental aspects of plant tissue and organ culture Reinert, J. and Bajaj, Y.P.S. (eds), Springer - Verlag, Berlin. pp. 93

7. Bonga, J.M. and Durzan, D.J. (1982).
In: "Tissue culture and forestry",
Nishaff, M. (ed.)
Dr. W. Jank Publishers, The Hague. P.P. 93

8. Boxus, Ph.; Quoirin, M. and Laine, J.M. (1977).
In: "Applied and fundamental aspects of plant cell, tissue and organ culture", Reinert, J. and Bajaj, Y.P.S. eds),
Springer - Verlag, Heildelberg, and New York
pp. 130-248

9. Chaturvedi, H.C. and Mitra, G.C. (1974).
Hort. Sci., 9, 118-120

10. Gamborg, O.L.; Miller, R.A. and Ojima, K. (1968).
Nutrient requirements of suspension culture of soybean root cells.
Exp. Cell Res., 50, 151-158

11. Gamborg, O.L., and Shyluk, J.P. (1981).
In: "Plant tissue culture method and applications in agriculture".
Thorpe, T.A. (ed.), Academic press pp 39-40

12. Gautheret, R.J. (1940).
Nouvelles recerches sur le bourgeonnement du tissu cambial d' ulmus campestris cultive.
In vitro CR Acad. Sci. Paris., 210, 744-746

13. Harada, H. (1975).
J. Hort. Sci., 50, 81-83

84

14. Hildbrandt, A.C. and Riker, A.J. (1953).
Influence of concentration of sugars and polysacharides on callus tissue growth *in vitro*.
Am.J. Bot; 40, 66-76

15. Huang, Li-chan and Murashige, T. (1976).
Plant tissue culture media: Major constituents their preparation and some applications.
TCA Manmeal, 2 (1), 539-548

16. Joley, E. (1969).
Pistachio. *In*: *Handbook of North American nut trees*., (R.A. Jaynes, ed) Knoxville, Northern Nut Growers Association, 348-361

17. Jones, O.P.; Hoo od, M.E. & O' Farrell, D (1977).
J. Hort. Sci., 52, 235-238

18. Magg, D.H. (1982).
An introduction to pistachio growing in Australia.
CSIRO Australia.

19. Murashige, T. and Skoog, F. (1962).
A revised medium for rapid growth and bioassay with toabacco tissue cultures.
Physiol Plant., 15, 473-497

20. Murashige, T. (1977).
Manipulation of organ initiation in plant tissue cultures.
Bot. Bull. Academia Sinica, 18, 1-24

21. Nickell, L.G. and Martzki, A. (1970).
Plant Cell Physiol., 11, 183-185

22. Optiz, K.W. (1975).
The Pistachio nut, University of California,
Division of Agricultural sciences, leaflet 2279,
Berkeley

23. Optiz, K.W. (1976).
Training young pistachio trees. University of California
Division of Agricultural sciences, leaflet 2961, Berkeley

24. Pannertier, C. and Lanand, C. (1976).
Fruits, 31, 739-750

25. Radojevic, L.; Vujicic, R. and Neskovic, M. (1975).
Z. Pflanzenphysiol. 77, 33-41

26. Reinert, J. Bajaj, Y.P.S. (eds), (1977).
In: "Plant Cell, tissue and organ culture".
pp. 94-95.
Springer - Verlag Berlin

27. Reynolds, T.F. and Murashige, T. (1979).
A sexual embryogenesis in callus cultures of palms.
In vitro, 15, (5), 383-387

28. Rao, P.S. and Bapat. V.A. (1978).
Can. J. Bot., 56, 1153 - 1156

29. Schenk, R.V. and Hilbrandt, A.C. (1972).
Medium and technique for induction and growth of
monocotyledonous and dicotyledonous plant cell cultures.
Can. J. Bot., 50, 199-204

30. Sommer, H.E. and Linda, S.C. (1981).
In: "Plant tissue culture methods and application in agriculture Thorpe, T.A. (ed.)
Academic Press, London, pp. 349

31. Sondahl, M.R. and Sharp, W.R. (1977).
Z. Pflanzenphysiol., 18, 395-408

32. White, P.R. (1963).
"The cultivation of Animal and Plant Cell"
2nd ed. Ronald Press Co., New York

33. White house, W.E. (1957).
The pistachio nut - a new crop for the Western United States.
Economic Botany, 11, 287 - 321

34. Yie, S.Y. and Liaw, S.I. (1977).
In vitro, 13, 564-568

35. Yokoyama, T. and Takenchi, M. (1976).
Phytomorph., 26, 273-275