PN ABM-708

REGENERATION & CLONAL PROPAGATION OF PISTACIA VERA BY TISSUE CULTURE

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PROJECT No. 936-5542

ANNUAL REPORT

(FROM 1-10-87 TO 30--9--88)

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FOOD TECHNOLOGY & FERMENTATION DIVISION
P.C.S.I.R. LABS.
FEROZEPUR ROAD LAHORE. (PAKISTAN)

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FIRST ANNUAL REPORT OF THE PROJECT # 936-5542
"REGENERATION OF PISTACHIA VERA".

The project was initiated on the 1st October, 1987. Recruitment of the workers was completed and the following were appointed on the dates mentioned against their names:

1.	Mr. Tariq Iqbal Shah M.Sc. (Botany)	Research Geneticist	15.10.87
2.	Mr. Zaheer Ahmad, M.Sc. (Chemistry)	Research Chemist	15.10.87
3.	Mr. Zamurd Hussain, B.Sc.	Junior Experimental Officer	15.10.87
4.	Miss Fehra Zafar M.Sc. (Botany)	Junior Experimental Officer	1.11.87
5.	Mr. Mujtaba Gilani, F.Sc.	Laboratory Assistant	1.10.87
6.	Mr. Naveed Haider	Laboratory Attendant	15.10.87

The following joined from the PCSIR Laboratories:

1.	Dr. Nasreen Zaidi (Botany)	Co-Principal Investigator
2.	Mrs. Shaista Jabeen M.Sc.(Chemistry)	Junior Scientific Officer
3.	Mr. Athar Hussain M.Sc. (Botany)	Ph.D. student

Services of M/s. Tariq Iqbal Shah and Zamurd Hussain were terminated after 7 and 3 months respectively due to their unsatisfactory performance. Mr. Mujtaba Gilani resigned due to his family circumstances and Miss S. Ayesha Hassan replaced him.

The following colleagues have submitted their synopsis for registration for Ph.D. studies:

1. Mr. Zaheer Ahmad) Copies of the synopsis
2. Mr. Athar Hussain) are attached
3. Mrs. Shaista Jabeen)

Laboratory facilities including four incubation rooms, one inoculation chamber, two rooms for media preparation with working bench facility were created during first year of the project. Equipment required for medium preparation, sterilization, inoculation and incubation of the explants was purchased and installed.

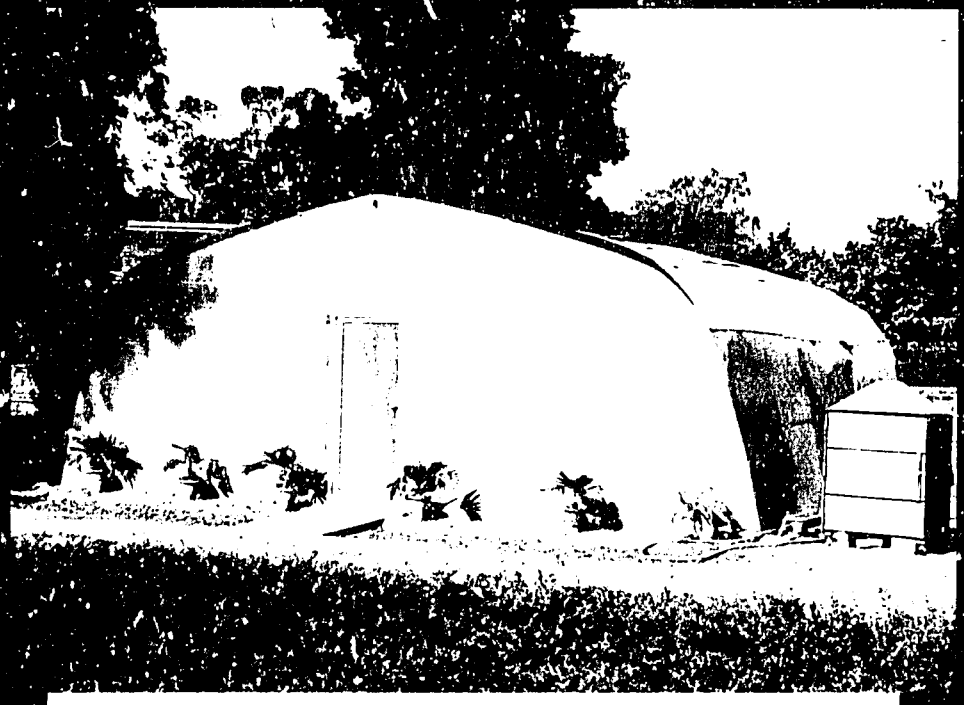
Two spacious rooms were furnished and handed over to the research staff.

Fabrication of Green Houses

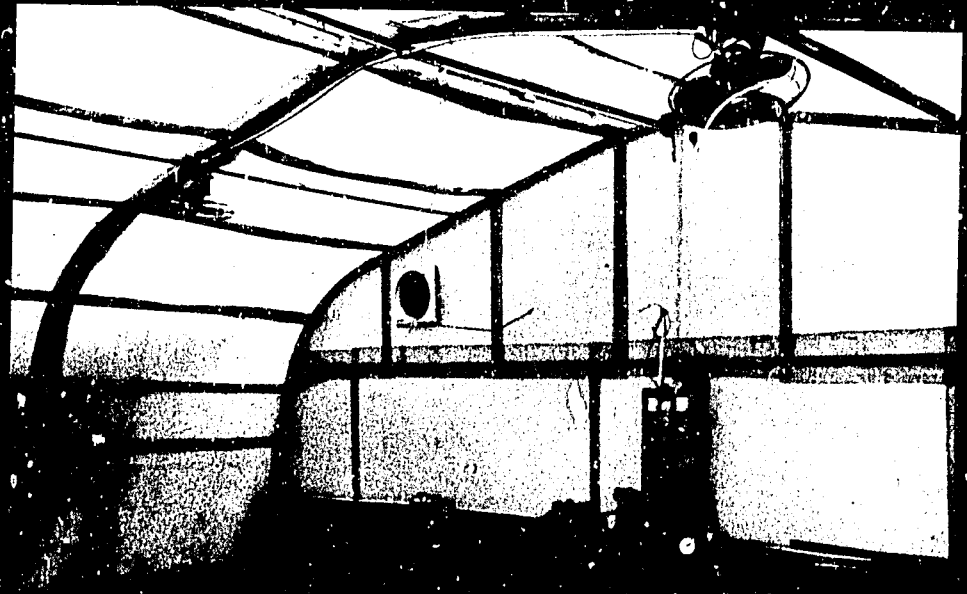
Two green houses 22' x 28', one covered with fibre glass and the other with double glass sheets were fabricated. These are fitted with air-conditioners, heaters, fans, humidifiers and other units for precise control of environmental conditions (temperature, light and humidity). All the units installed in the green houses are locally available so repair or replacement will not be a problem (photographs attached).

Lists of equipment and chemical to be purchased were prepared, tenders were floated and comparative statements were prepared. Final list for the purchases was prepared on the basis

GREEN HOUSE (FIBRE GLASS)

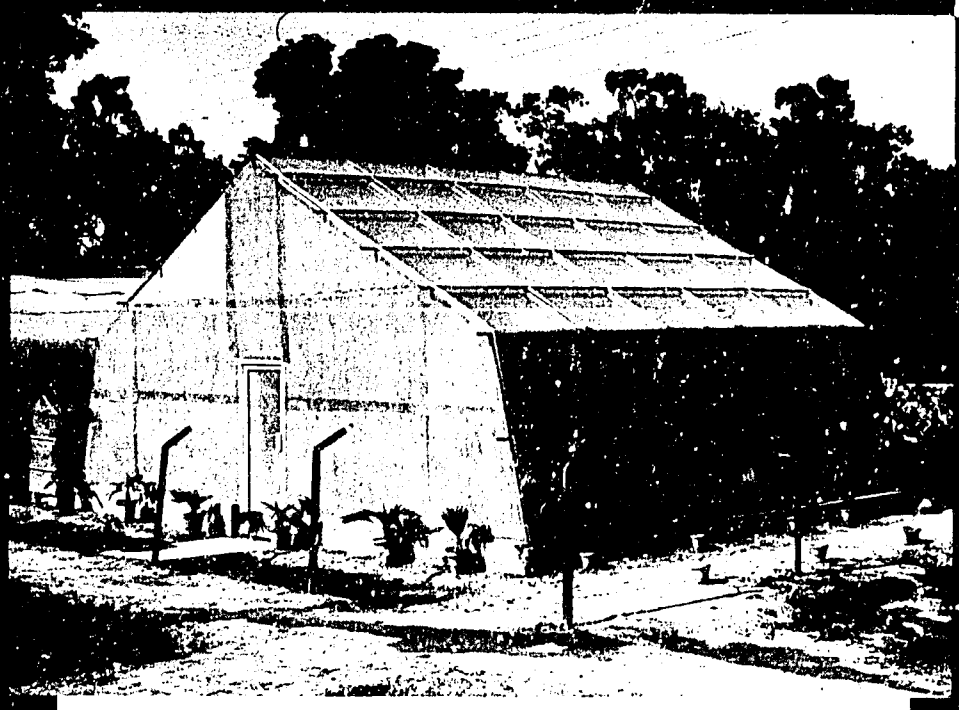


EXTERIOR VIEW



INTERIOR VIEW

GREEN HOUSE (GLASS)



EXTERIOR VIEW

INTERIOR VIEW

of the instructions contained in the contract document and submitted to US AID office, Islamabad.

Provision of Healthy Seedlings

The first step consisted of providing healthy seedlings from aseptically germinated seeds for the studies mentioned below:

a) somatic embryogenesis
b) rejuvenation work
c) development of new varieties
d) clonal propagation

Seeds of *Pistacia vera* served as the source of explants. The seeds were obtained from the Department of Agriculture, Quetta, Pakistan. Results of aseptic culture of healthy pistachio seedlings indicated that physical and chemical treatments affected germination of the seeds as well as health/vigor of the seedlings.

1. Disinfection of the seeds with 0.1 % (w/v) $HgCl_2$ solution for 15 minutes effectively controlled contamination and improved germination response.
2. Removal of seed coat resulted in mechanical injury which reduced supply of nutrients from cotyledon to young plantlet. Polyphenolic discharge, during vigorous growth of the cotyledons, inhibited growth of the seedling.
3. Chilling at $4^{o}C$ helped in breaking dormancy of the seeds. Germination response of the seeds with seed

coat, after chilling for 48 hrs and incubation at 25 ± 2°C was maximum (75 %).

4. Incubation at 15°C, without chilling, resulted in 80 % germination of the seeds.

5. Seeds cultured on inert substrate or nutrient media with or without supplementation with growth regulators affected size of the root, shoot and number of leaves.

   a) Seedlings grown on media supplemented with hormones grew vigorously. Length of shoot and size of leaves was maximum in the presence of the growth regulators. Root growth and number of leaves was more in hormone free media.

   b) Supplementation of the nutrient medium with BA, NAA and Kin individually or in combination markedly affected size and number of leaves and stem. 4 mg/l BA plus 1 mg/l each of Kin and NAA induced multiple shoot formation with a diameter of 30-35 mm and leaf size (lxw) 40x30 mm.

   c) Macro and micro nutrients of four different media, as reported by Murashige and Skoog (1962) Lloyd and McCown (1980), Driver and Kunijuki(1984) and Eriksson (1965), had minor effect on seed germination and explant survival percentage or increase in the size of the explant. Woody Plant Medium composition was most suitable for explant

survival, Ms, WP and EK media induced maximum increase in the size of the explant. MS, WP and DK media were equally effective for seed germination. B_5 medium had little effect on germination/survival percentage and increase in the size of the explant.

Selection of Suitable Explant Material

Explants obtained from young seedling were cultured on MS medium containing BA (0.1-2 mg/l) and NAA (0.1-1.5 mg/l). Tissues of the cotyledonary explants were found to be most active. Cotyledonary explants formed calli, roots and embryoids. Roots and callus were formed by cotyledonary stalks while roots of seedlings formed only calli. Slides prepared by free hand cut sections of the cultured cotyledons showed heart shape embryos with definite vascular axis and developing cotyledonary lobes. Further development of the embryos was restricted due to embryos inhibitory effect of polyphenolic substances. The study indicated that cotyledons of the seedlingscan be safely used as explants for somatic embryogenesis and clonal propagation by tissue culture and for development of new varieites by cell culture technique.

Clonal Propagation by Tissue Culture Method

Cotyledons of seeds grown on culture media containing auxins and cytokins in different concentrations formed calli. Low concentration of 2,4-D or IAA (i.e. 1-2mg/l) induced callus

formation but the texture was poor. Combination of 2,4-D with IAA, or NAA, KIN and/or BAP induced, active callogenesis and formed fair textured calli. Fair quality texture was also formed in the presence of relatively high concentration of 2,4-D (4 mg/l) but callus induction was delayed. Similarly addition of GA_3 retarded callus induction but the callus formed was of fair texture.

Cotyledonary explants actively responded to a combination of 2,4-D and KIN (2 mg/l each). Calli were nodular and white and there was no browning of the tissue. Callogenesis was induced at a slow rate when the medium contained 2 mg/l BAP and 2 mg/l of 2,4-D. Browning of the tissue was also evident. 4 mg/l NAA also actively induced callus formation along with adventitious roots emerging directly from the cotyledons.

Addition of NH_4Cl (100 mg/l) to the medium containing 2 mg/l of BAP, KIN and 2,4-D affected texture of the calli and regeneration potential. Calli were white, nodular, compact or friable, but no embrogenesis was noted whereas Torpido shaped embryos were observed in a medium without NH_4Cl.

Development of New Varieties by Cell Culture

Production of more and friable calli was the first step to initiate cell culture of *P. vera*. Cotyledons of growing seedling were kept on full and half strength MS media supplemente with various auxins and cytokinins, added singly or in combinatio Response regarding colour, texture and callus formation was observed.

Full strength MS medium was most suitable for obtaining good quantity calli whereas half strength medium was effective for improvement in friability of the calli.

Variety of explants, cotyledons, embryonic axis, seed coat, shoot tissue and mesocarp tissue were used for callus formation. Best results were obtained with cotyledonary explants while mesocarp tissue also formed callus after 3 or 4 subcultures. Subculturing helped in overcoming the inhibitory effect of polyphenolic discharge from cut ends of the explants.

Experiments were conducted to obtain most suitable physical and chemical factors for the formation of highly friable calli. This is being extended to the introduction of callus culture to suspension medium.

Anatomical Analysis of the Explants Cultured *In Vitro*

Effect of different hormonal combinations on juvenile explants was anatomically analysed. Sections of explants were prepared by microtomy and free hand cutting. Calli were observed by smear method, Induction of trachial element or any morphological change in parenchymatous tissue were detected by observing the slides under microscope after passing through specific staining techniques. Photographs of some of the results are recorded and interpreted individually.

REGENERATION THROUGH TISSUE CULTURE

PLAN OF WORK

1. Induction of callus from juvenile explants of seedling.
2. Induction of Somatic embryogenesis and/or organogenesis from variety of calli

ABBREVIATIONS

IBA	Indolebutyric acid
KIN	Kinetin
NAA	Naphthalene acetic acid
BAP	6-Benzyl amino purine
IAA	Indole-3-acetic acid
MS	Murashige, T. & Skoog, F. (1962)
B_5	Gamborg *et al*. (1968)
WP	Woody Plant medium
Ek	Erickson's medium
Dk	Driver and Kunijuki

RESULTS

Seeds of P. vera (with and without seed coat) were disinfected, before culture in vitro, by soaking in 0.1 % (w/v) mercuric chloride solution containing Tween 20 at a rate of 5-6 drops per 100 ml of the disinfectant. It is evident from table 1 that soaking of the seeds without seed coat for 10 minutes was most suitable for controlling contamination. Only 10 % of the explants become infected during incubation whereas germination response was also fair. The seeds with seed coats took more time (15 minutes) for disinfection and the rate of contamination was somewhat more i.e. 20 %. However, there was improvement in the germination. The drawback in either case was that health of the ascentically grown seedlings was quite poor.

The effect of disinfectants on growth of seedlings and their health is presented in table 1. The seed coat seems to maintain some compressional 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 the cotyledons, due to excessive water absorption, in case of seeds with seed coats

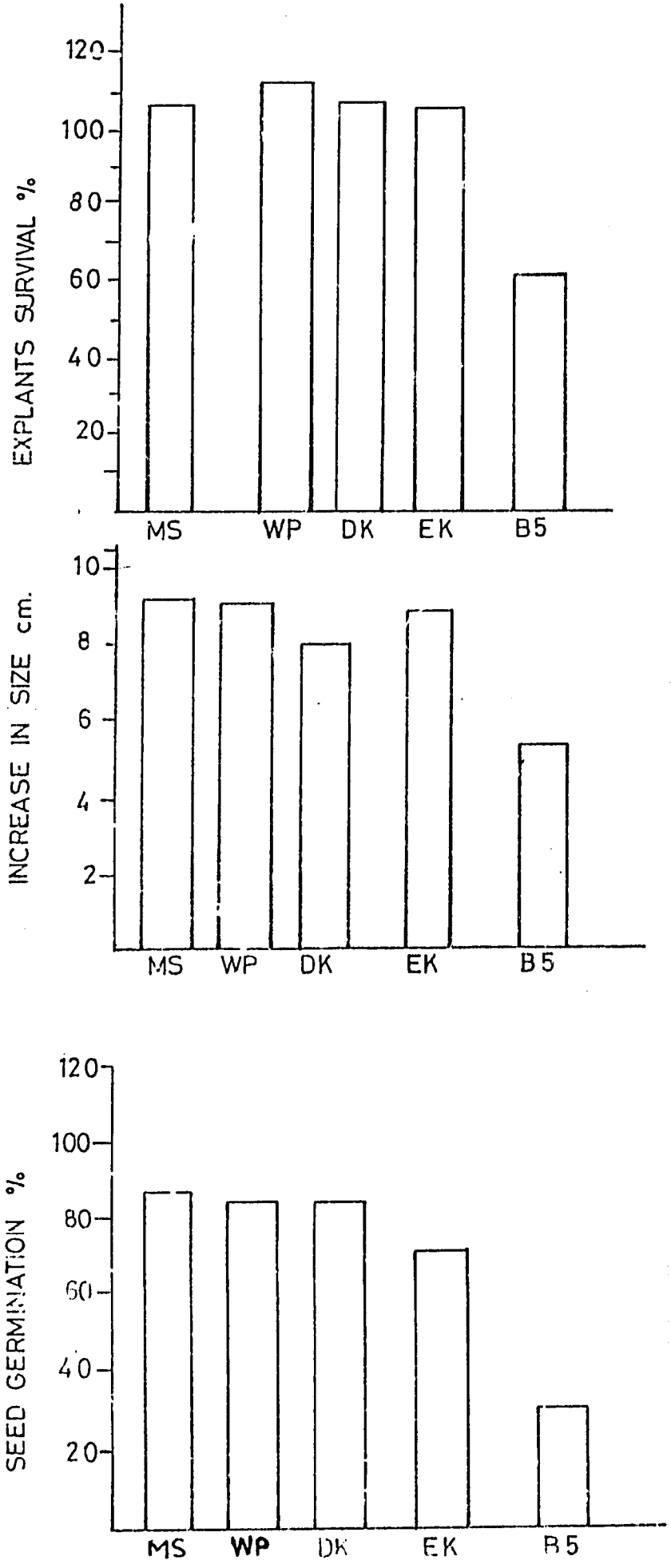
caused minute injuries along with the abaxial surface of the cotyledons which resulted in browning of the cotyledons and of the medium. This adversely affected growth of the seedlings (table 2).

It is evident from table 5 that chilling at $4^{o}C$ helped in breaking dormancy of the seeds. Germination response of the seeds with seed coat, after cold treatment for 48 hours and incubation at $25\pm2^{o}C$ was maximum (75 %).

Suitable salt compositions for improving the health of the seedlings were selected by screening. Seeds were grown on MS, B_5, WP, DK and Ericksion salt medium and their growth, increase in size and callus induction capacity showed that WP and MS media were the best for Pistachio tissue. MS media was still better than WP medium as callus produced on MS medium was found to be more nodular and compact. So, this medium was used in the rest of the studies (Fig.I). Sucrose at the rate of 3 % and Agar at 0.7 % were also added to the medium.

Improvement in the 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 the medium containing 1.0 mg/l BA and 0.25 mg/l NAA. 47 % germination was observed in a medium containing 1 mg/l KIN and 0.25 mg/l NAA, whereas a combination of 4 mg/l BA,

Fig. I. THE COMPARATIVE PERFORMANCE OF JUVELINE P. VERA TISSUE EXPLANT WHEN PLACED ON 5 DIFFERENT SOLID BASAL MEDIA.



1 mg/l NAA and 1 mg/l KIN further enhanced the germination percentage upto 55 % and improved the health of seed.

There was a marked increase in the size of the leaves and diameter of main shoots of the seedlings grown on medium containing hormones. In case of medium containing BA alone, BA + NAA or KIN + NAA single shoots with 1.0, 1.5 and 2.0mm diameter respectively were produced. Multiple shoots with 20-30 mm length were induced in the presence of BA + NAA + KIN. The size of the leaves was approximately four times more than those grown on medium without hormones (table 3).

Explants of seedlings root, shoot and cotyledons cultured on media containing different concentrations of BA and NAA exhibited different morphological behaviour (table 4). 1 mg/l each of NAA + BA induced callus formation in explants of cotyledons, formed adventitious roots (Fig.4) on medium containing 0.1 mg/l BA + 1.5 mg/l NAA, while globular embryoids were observed on the medium containing 2 mg/l BA and 0.1 mg/l NAA.

2,4-D was supplied to the cotyledon explants at a rate of 2,4,6,8,10 mg/l. 2mg/l of 2,4-D in the medium was found to be the best for callus formation and 2,4-D 2 mg/l in combination with KIN 2 mg/l was found best, as cotyledons produced granular callus in this medium (table 6).

Callus was observed to be more embryogenic when NH_4Cl was also supplied in the medium (table 7), but no true embryoid formation has yet been observed in the medium containing NH_4Cl.

GENERAL DISCUSSION

Pistacia vera L. is one of those plants which are relatively new in plant tissue culture technology. So, to start with it was necessary to establish sterilization techniques and the best suited culture medium and culture conditions. To achieve this goal a number of preliminary experiments were conducted on the basis of which it was established that 0.1 % (w/v) mercuric chloride solution was the best for effective sterilization and it did not damage the seeds or the embryo. 10 minutes soaking of the seeds without seed coat was best to get the highest germination rate (47 %) whereas seeds with seed coats took 15 minute for disinfection and showed improvement in the rate of germination Seed coat also played an important role and experiments have shown that seeds without seed coat either died during sterilization or they absorbed more water which caused breakdown of the cotyledonary stalks which proved harmful for the health of the emerging embryo.

Among commonly employed salt mixture Woody Plant medium and Murashige and Skoog medium were better for callus formatior from stem, root, leaf or cotyledons. The explant attained muct larger size, approximately 3-4 times the original explant, wher woody plant medium was used but the amount of callus produced was less and callus was not granular; while explants on MS medium showed some increase in the size but the production of callus was more and it was white and nodular.

A combination of BA (2 mg/l) and NAA (0.1 mg/l) produced very good results. Here embryogenesis was noticed and in hand cut sections clear heart-shaped embryos were observed. BA 4.0 mg/l + KIN 1.0 mg/l + NAA 1 mg/l was found best for growth of healthy seedlings.

Shortcomings

Non-availability of chemicals and equipment was the major hinderance in accelerating pace of work.

TABLE-1: EFFECT OF MERCURIC CHLORIDE TREATMENT ON EFFECTIVE DISINFECTION OF SEEDS

Nature of seeds	Soaking time (min.)	Contamination (%)	Germination response	Remarks
Without seed coat	3	100	+	Germination was accompanied with fungal contamination.
	10	10	+++	Contamination controlled.
	15	-	-	Seeds died
With seed coat	10	70	+++	Germination restricted due to fungal attack.
	15	20	+++	Germination improved.
	20	10	+	Germination delayed. Seeds die off

+ Poor
+++ Fairly good
- Nil

TABLE-2: EFFECT OF INOCULATING *P.vera* SEEDS, WITH/WITHOUT SEED COATS, ON GERMINATION

Parameters studied	Seeds with seed coat	Seeds without seed coat
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 cotyledons was broken.
Browning of tissue	Coloring substances were discharged from the seed coats which do not inhibit normal growth of seedling.	- Cotyledons also grow with seedling. - Vigrous increase in size of cotyledons without seed coat protection causes numerous minor injuries on their adaxial sides. - As a result polyphenols were discharged which were injurious to seedling health.

TABLE-3: EFFECT OF GROWTH REGULATORS ON GERMINATION RATE AND SEEDLING VIGOUR

Growth Regulators GRs	Concentration of GRs mg/l	Germination %	Shoot		Leaf size L x W mm
			Thickness mm	Number	
BA	1.0	42	1.0	Single	10x7
BA + NAA	1.0 + 0.25	42	1.5	Single	15x15
KIN + NAA	1.0 + 0.25	47	2.0	Single	25x15
BA + KIN + NAA	4.0 + 1.0 + 1.0	55	3.0-3.5	Multiple	40x30

TABLE-4: EFFECT OF VARIOUS CONCENTRATION OF BA AND NAA ON MORPHOLOGICAL RESPONSE OF *P.vera* SEEDLING EXPLANTS

BA	+	NAA	Explant	Morohological response
1 mg/l	+	1mg/l	Cotyledon	Callogenesis
			Cotyledonary stalk	Rhizogenesis
			root	Callogenesis
1 mg/l	+	0.1mg/l	Cotyledonary stalk	Callogenesis
0.1	+	1.5	Cotyledon	Rhizogenesis
2.0	+	0.1	Cotyledon	Callogenesis + embryoid

TABLE-3: EFFECT OF TEMPERATURE ON BREAKING DOWN OF DORMANCY AND GERMINATION PERCENTAGE

	Group of seeds	Germination % after chilling at 4°C					Remarks
		6 hrs.	12 hrs.	24 hrs.	48 hrs	60 hrs.	
Sterilization and inoculation before temperature treatment	With seed coat	30	35	50	70	70	Chilling treatment is necessary to break down the dormancy of the seeds. It may be done before or after sterilization and inoculation. The treatment was found less effective before 48 hrs. interval is complete. Chilling after 48 hours did not improve rate of germination. Seeds without seed coat were less successful in germination
	Without seed coat	10	28	30	30	30	
Sterilization and inoculation after temperature treatment	With seed coat	30	35	55	75	70	
	Without seed coat	10	20	20	35	35	

Salt composition = MS medium

Sterilization agent = $HgCl_2$ 0.1 % + 15 min. soaking.

Incubation temperature = $25 \pm 2^{o}C$

TABLE-5: EFFECT OF 2,4-D & DIFFERENT CYTOKININS ON THE GROWTH OF TISSUE EXPLANT IN VITRO

Growth regulators mg/l							Browning Tissue	Tissue response		
Auxins										
2,4-D	IAA	IBA	NAA	BAP	KIN	2iP		Callus	Regeneration	General Remarks
2.0	-	-	-	2.0	-	-	++	+	-	Slow growth of callus.
2.0	-	-	-	-	2.0	-	-	++++	-	Actively growing callus nodular white is color.
2.0	-	-	-	-	-	2.0				
2.0	-	-	1	1	-	-	Browning appeared in the beginning then finished	+++	Root	Roots grew on the cotyledons
2.0	1	-	-	-	2	-	-	++	-	Slow growth.
-	-	-	4	-	-	-	-	++++	Root	Vigrous growth of roots from cotyledons

Medium used = Murashige and Skoog
Sucrose = 3 %
Agar = 0.7 %

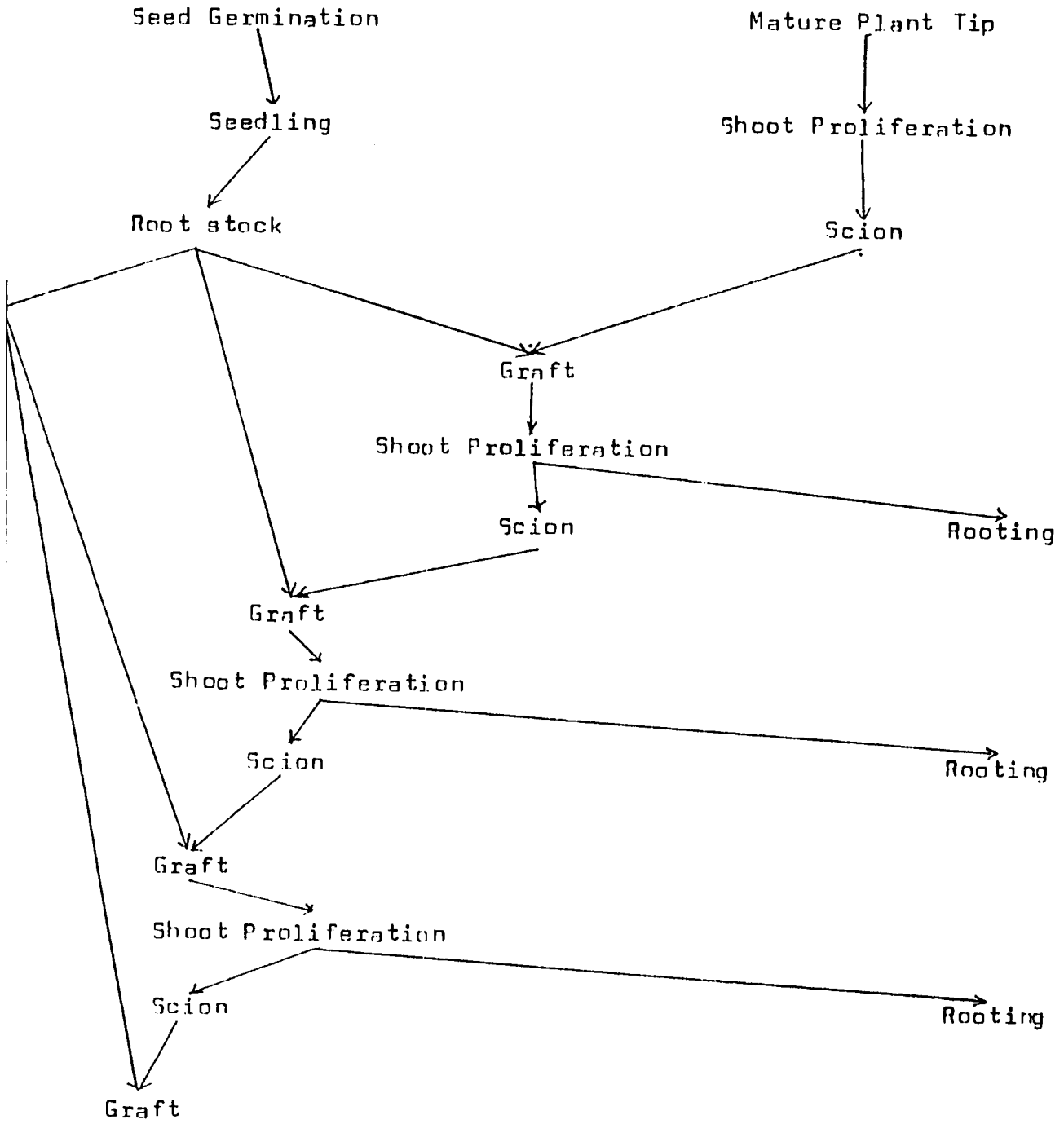
TABLE-7: EFFECT OF NITROGEN SUPPLEMENTATION ON THE TISSUE RESPONSE in vitro

Growth regulators mg/l									Tissue response			
BAP	KIN	Zip		2,4-D	IAA	IBA	NAA	NHCl mg/l	Callus inoculation	colour of callus	Regeneration	General remarks
2.0	-	-	-	-	-	-	0.1	-	+++	White	Embryo	Embryo were developed upto torpido shape
2.0	-	-	-	-	-	-	1	-				
-	2.0	-	-	2.0	-	-	-	-	+++	White	-	Friable callus
2.0	-	-	-	-	-	-	1	100				
-	2.0	-	-	2.0	-	-	-	100	+++	White	-	Nodular compact
-	2.0	-	-	0.2	-	-	-	-	+++	White	-	Friable
-	2.0	-	-	0.2	-	-	-	100	+++	White	-	Nodular less in quantity.

+++ Very good

REJUVINATION OF MATURE P.Vera BY MICROGRAFTING TECHNIQUE

WORKING PLAN



Kernal of Pistacia vera provided by the Horticulture Department of Baluchistan Government or purchased from the local market were used during the studies. The mature tips were also supplied by the Horticulture Department.

The seeds were found to be heavily contaminated. These were decontaminated first by dipping in 90 % ethyl alcohol for 1 minute and then by soaking in solutions of mercuric chloride or calcium hypochlorite.

Effect of Temperature on Germination of Seeds

Decontaminated seeds were kept on wet cotton in a 4 inches petri plate at the rate of 10 seeds per plate and kept in the dark at ambient temperature. The seeds failed to germinate under these conditions. On gradually lowering the temperature from 35°C to 15°C it was found that the rate of germination improved with a decrease in temperature. It was 15 % at 25°C, 60 % at 20°C and 80 % at 15°C(table 8).

Effect of Growth Promotors and Nutrients on Growth of the Seedling

Germinated seeds were propagated on MS and WP nutrient media with or without growth regulators. Effect of these changes on length of the stem and root and number and area of leaves was studied. It was noticed that the germination of the seeds was not affected by growth regulators and nutrients provided in the medium. But once germination was initiated further growht of the seedling was greatly affected by the media composition. Germination of seeds was noticed after 4 days of incubation and all the seeds germinated

TABLE-8 : EFFECT OF TEMPERATURE ON SEED GERMINATION

Temperature	Batch No.	Number of seeds	Germination	Percentage of germination
35°C	1	5	0	
	2	5	0	
	3	5	0	0
	4	5	0	
	5	5	0	
30°C	1	10	1	
	2	10	2	
	3	10	0	10
	4	10	1	
25°C	1	10	2	
	2	10	1	
	3	10	1	15
	4	10	2	
20°C	1	10	7	
	2	10	6	
	3	10	5	60
	4	10	6	
15°C	1	10	8	
	2	10	8	
	3	10	7	80
	4	10	9	
	5	10	9	

within 7 days. The rate of growth in case of the seeds kept in the dark even after germination, was slower than those which were transferred to light. The food reserve in the cotyledon invariably exhausted after 3 weeks, and the seedlings started dying if not transferred to fresh nutrient medium.

Table 9 shows a study conducted on seedlings with or without cotyledons cultured on media without nutrients, with nutrients alone or nutrients supplemented with growth regulator. The changes in length of root, shoot and number and area (length x width) of young leaves till the seedlings showed signs of deterioration/ necrosis were noted. Growth of seedlings with cotyledons on sterile moist cotton was minimum. Lengths of stem and root were 1.5 cm and 3.5 cm respectively at the end of the third week. At the same time an average of five leaves (2-5 mmL x 2.5mmW) per seedlings were present. In the fourth week shoot tip necrosis appeared.

Seedlings without cotyledons grown on hormone free nutrient medium showed some improvement in the length of stem and root which was 2.5 cm and 5 cm respectively while average leaf number was 8 per seedling with length and width of lamina 1.0 and 0.7 cm respectively. Growth of cotyledon free seedlings again showed some signs of improvement on hormone supplemented nutrient medium. Average shoot and root lengths, after three weeks, were 4 and 3 cm respectively. But average leaf count was lesser i.e. 5 leaves (2.5 cmL x 1.7cmW) as compared to 8 in case of cotyledon less seedlings grown on nutrient medium only.

Batch of seedlings with complete parts grown on simple nutrient

TABLE - 9 : EFFECT OF NUTRIENTS AND GROWTH REGULATOR ON SEEDLING GROWTH

	Wet cotton only				Nutrients without growth regulator								Nutrients with growth regulator							
	With cotyledon				Without cotyledon				With cotyledon				Without cotyledon				With cotyledon			
	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week
Length of stem	1-2 mm	1cm	1.5 cm	Plant starts drying	1-2 mm	1cm	1.5 cm	2.5 cm	1-2 mm	1.5 cm	4cm	Necrosis appeared after 23-27 days	2-3 mm	1.5 cm	4cm	Necrosis appeared in 16-19 days	2-3 mm	1.5 cm	5cm	Necrosis started
Length of root	2-3 mm	2cm	3.5 cm		2-3 mm	3cm	4cm	5cm	2-3 mm	4cm	6cm		2-3 mm	2cm	3cm		2-3 mm	2cm	3cm	
Number of leaves	0	3	5		0	3	5	8	0	5	8		0	3	5		0	3	5	
Length of leaves	0	2-3 mm	2-5 mm		0	3-7 mm	3-7 mm	1cm	0	3-7 mm	1.5cm		0	1.5 cm	2.5 cm		0	1.5 cm	2.5 cm	

Necrosis appears in 25-28 days

medium and hormone supplemented nutrient medium showed marked difference in length of roots and number and size of leaves. On simple nutrient medium stem and root length were 4 cm and 6 cm, average leaf count was 8 with lamina size 1.5 cmL x 0.9 cmW. Shoot and root length on hormone supplemented media were 5 cm and 3 cm respectively but number of leaves per plant was 5. The leaves were more extended (2.5 cmL x 1.8 cm W). In first three conditions, fourth week was disastrous for almost all the seedling batches with/without cotyledons, grown on inert or enriched media. On wet cotton the seedlings started drying while on nutrient medium shoot tip necrosis appeared irrespective of the condition for cotyledons intact or removed. Shoot tip necrosis appeared during third week of culture in cotyledonless seedlings growing on hormone supplemented nutrient medium.

Shoot Proliferation

Shoot proliferation was the major object of the study. Proliferation was attempted in case of both juvenile and mature shoots. Partial success was achieved in case of juvenile shoots. Plant regulators activated the buds in the axil of the leaves. A combination of BA (4 mg/l) and NAA (0.25 mg/l) activated the buds which resulted in 6-8 new stems from a single stem.

The work is in progress.

Problem Faced During Work

1. Necrosis which generally appeared in the second last intermode made it difficult to maintain the explant stock. As a result

of necrosis the healthy tips died and the axial buds became active. Necrosis again appeared when the plant attained the same height. This process was repeated till the death of the plant.

2. Browning problem was encountered when the seeds were decontaminated with calcium hypochlorite solution and not with mercuric chloride solution. However, the mature explant in both the experiments was killed due to browning. Subculturing was found to be partially effective.

REGENERATION THROUGH CELL CULTURE

Plan of Work

Stage 1	Induction of callogenesis
Stage 2	Initiation of suspension culture
Stage 3	Induction of embryogenesis and organogenesis
	Transfer to Green Houses

ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxy acetic acid
Kin.	Kinetin (6-furfuryl aminopurine)
IAA.	Indole acetic acid
NAA.	Naphtalene acetic acid
IBA.	Indole butyric acid
BAP (BA)	Benzyl amino purine
2iP.	2-Isopentenyl adenine
GA_3.	Gibberellic acid
MS	Murashige & Skoog medium
WPM	Woody plant medium
GR	Growth regulators

The main aims and objectives of the work of stage I were:

1) To induce callogenesis in different explants.

2) To induce friability in calli obtained.

3) To select combinations of growth regulators which would give good quantity calli.

4) To minimize browning.

5) To find out the number of subculturing that a callus would sustain.

6) To study the degree of sterilization achieved with different concentrations of $HgCl_2$, $Ca(OCl)_2$, NaOCl, and

7) To study the effect of concentration of inorganic ions.

The main emphasis had been to obtain calli that were good in quantity and colour by using different concentrations of growth regulators. The combinations used for this purpose are:

a) salt mixture + auxin only

b) salt mixture + auxin + cytokinin

c) salt mixture + cytokinin only

Table 1 encompasses results of the experiments that were conducted to obtain good quantity calli with good friability.

The results of the studies with media containing full strength MS salt mixture plus 3.0 per cent sucrose + 0.7 % agar, half strength MS salt mixture plus other ingredients or full strength MS salt mixture plus 1.0 per cent sucrose plus 0.7 per cent agar, after supplementation with different concentrations of

hormones are denicted in tables 10-12. It is evident that quantity and friability of calli varied when full strength MS salt mixture was supplemented with various concentration of hormones. The best(highly friable and in good quantity calli) results were obtained when 5 mg/l NAA or 2 mg/l each of 2,4-D plus KIN or 0.5 2,4-D + 2.0 IAA + 0.5 KIN (mg/l) were added to full strength MS salt mixture (table 10).

Reduction in the strength of MS salt mixture to half also gave highly friable and in good quantity calli when the medium contained 2 mg/l each of 2,4-D and KIN (table 11). Reduction in the amount of sugar from 3 per cent to 1.0 per cent adversely affected both quantity as well as friability of calli (table 12).

Replacement of MS salt mixture by full strength WPM resulted in the formation of friable calli in fair quantity only. Reduction in the strength to half resulted in a decrease in the quantity of the calli as well as their quality (table 14).

It is evident that rate of initiation of senescence due to browning of callus was different for different batches containing different concentration and combinations of growth regulators and salt mixtures. Histograms (A-C) show the time taken by different calli to become "brown". MS medium in full strength and half strength was employed in all the batches shown in histogram A and B, whereas woody plant medium (full strength) was employed in the batches in histogram C.

Callus initiation was successfully obtained from cotyledon, mesocarp, embryonic axis, seed coat and shoot. Some of the

concentrations and combinations of growth regulators which induced calli in different explants are given in table 15.

Initiation of cell suspension culture has only recently been undertaken. Some of the work done has been tabulated in table 16.

GENERAL DISCUSSION

The work that has so far been accomplished mainly centered around the study of chemical manipulations for the appropriate selection of growth regulators in proper concentration and combinations. Studies also involved the selection of salt mixtures and the impact produced by varying their strengths. Following parameters were studied.

1) Production of Good Quantity Calli

The main purpose of the study was to obtain good quantity calli. This was accomplished by trying different concentration and combinations of growth regulators with full strength Murashige and Skoog (MS) medium (table 10) and by repeating results mentioned in table 10 with half strength MS medium (table 2), with full strength Woody Plant Medium (table 13) and with half strength WPM (table 14).

Table 10 clearly shows that maximum quantity (++++) of calli was produced in batches No.56, 17 and 9 (table 10). The quantity of calli in batches No.30, 33,70,80,87, 77, 82,49,72,23 and 73 was slightly lesser than batches No.9,17 and 56 (table 10). The quantity was still less in batches No.19,69,5,53,15,28,76 and 84.

The concentration and combinations of growth regulators which gave optimal yield (table 10) were then tried with half strength MS medium (table 11). It can be judged from the results that the quantity of callus produced in batch No.86 and 113 (table 11) corresponded with the results of batches No.9 and 19 (table 10). The quantity of callus formed in batches No.119 and 127 (table 11) was more than batches No.31 and 16 (table 10). The quantity of calli in batches No.120, 122, 123, 124 and 117 (table 11) was lesser than that observed in case of batches No.32, 82, 83, 87 and 23 (table 10). It is evident from table 11 that the quantity of callus formed decreased when half strength MS medium was employed.

The positive results of table 10 were then repeated with full strength woody plant medium. Results included in table 13 show that quantity of callus formed remained the same in batch No.96 and 98 which corresponded to batches No.23 and 76 respectively (table 10). The quantity of callus formed in batches No.90, 91, 92 and 101 was lesser than that in batches No.9, 15, 41 and 83 (table 10). Therefore it can be safely concluded that the quantity of callus reduced when woody plant medium (WPM) was employed.

The results of table 10 were then repeated on half strength woody plant medium. Table 14 shows that the quantity of callus was less in batches No.103A, 103B, 103C, 103D, 103E and 103F as compared to batches No.9, 19, 76, 56, 72 and 23 respectively (table 10). Reduction in the amount of sucrose from 3 to 1 %

resulted in a decrease in the production of callus (table 12).

These results indicated that full strength MS medium was most suited for obtaining optimum quantity of callus.

2) Friability

Calli formation in different batches as shown in tables 10-14 indicated that the growth regulators and salt mixtures produced profound effect upon the friability of the calli formed.

When MS medium in full strength was employed, the maximum (++++) friability was produced in batches No.9, 17 and 56 (table 10). Very good (+++) quality calli were obtained in batches 30, 33, 53, 19, 72, 74, 80 and 83 (table 10)and (++) quality calli were obtained in batch No.15, 32, 62, 70, 5, 23, 73, 77, 84 and 87 (table 10).

The results of table 10 were repeated with half strength MS medium,keeping all the other conditions constant (table 11). Friability of the calli formed in batches No.86 and 113 (table 11) was comparable with batches No.9 and 19 (table 10). There was a definite increase in friability in batches No.117, 119, 120, 122, 123, 124 and 127 (table 11). These results indicated that friability increased when the strength of MS medium was reduced to half but reduction in the concentration of sucrose had adverse effect (table 12).

The experiments with full strength woody plant medium (WPM) (table 13)showed that the friability in batch No.98 was better than batch No.76 (table 10). The friability in batches No.93 and 101 was comparable with batches No.33 and 83 (table 10) which deteriorated in batches No.90, 91 and 96 (table 13). It appears

from the results of table 13 that the friability was not improved when WPM (full strength) was employed.

A similar trend was noticed when half strength woody plant medium was employed (table 14).

3) Sources of calli

Callus formation was successfully achieved through cotyledon, mesocarp, embryonic axis, seed coat and shoot, tissues. Some of the significant results are recorded in table 15. Best calli were generally achieved through cotyledon. The most interesting observation was the induction of callogenesis through mesocarp tissue.

4) Browning of callus

Senescence due to browning had been a major problem for the dedifferentiated cells. Time period, from initiation to complete browning, varied with different concentration and combinations of growth regulators.

Fig.1 clearly shows that the maximum period for the browning to overcome the callus was observed in batches containing only NAA. It took 13 weeks in case of batch No.56 (NAA 5 mg/l). and 12 weeks for batch No.53(NAA 2 mg/l). The time period for browning to overcome the callus was also delayed when 2,4-D + Kinetic or 2,4-D + Kinetin + NAA/IAA were employed. It took 10 weeks for batch No.19 (2,4-D(3mg/l) + Kinetin (2mg/l); 9 weeks for batch No.72 (2,4-D(3mg/l) + NAA (3mg/l) + Kinetin(3mg/l) and 6 weeks for batch No.17 (2,4-D(0.5 mg/l) + Kinetin (0.5mg/l)+ IAA (2mg/l). Although it took 10 weeks in batch No.19, it took

only 5 weeks in batch No.9 (2,4-D (2mg/l) + Kinetin (2 mg/l) where 2,4-D was reduced to 2 mg/l. It took 5 weeks for batch No.23 (2,4-D (0.2mg/l) + IBA (5mg/l) + BAP (5mg/l) + GA_3(0.5mg/l) and 4 weeks for batch No.33 (2,4-D (4mg/l).

When MS x ½ medium was employed (Fig.2) the "browning" time was not affected. It was 5 weeks in batch No.86 and 4 weeks each in batch No.119, 120 and 122 (see corresponding batches No.9, 33, 30, 74 respectively of Fig.1). The use of WPM in full strength also did not produce any significant variation in the "browning" time (Fig.3). The variation usually ranged between ± 1 week, when "available" results of MS x 1 (Fig.1) were compared with WPM x 1 (Fig.3). Browning overcame in 6.5 weeks in batch No.99 (2,4-D(4 mg/l) + NAA (2 mg/l) + Kinetin (2mg/l) and 101 (2,4-D (3md/l) + IAA (3mg/l) + Kinetin (3mg/l) + GA_3 (0.5 mg/l). It took 6 weeks in batch No.93 (2,4-D (4 mg/l).

It is very difficult to cross-compare the results of Figures 1, 2 and 3 as the results of corresponding batches are not available. More work is being done to this end.

By comparing the results given in tables 10 - 14 and Figures 1,2 and 3 we can select the following batches for taking the work into stage No.2.

1	Batch	No.9
2	"	19
3	"	56
4	"	53
5	"	30
6	"	33
7	"	17
8	"	23

9	Batch No.	72
10	"	73
11	"	80
12	"	83
13	"	87
14	"	86
15	"	82
16	"	127
17	"	90
18	"	93
19	"	101
20	"	103D

Work on stage No.2 of the plan of work has been initiated. ıtches with inoculum density varying from $1x10^4$ cells/ml ›atch No.757) to $4x10^4$ cells/ml (batch No.758 and 756) have ›en set up. Cell density has definately increased in batches ›.757, 758 and 756.

ACHIEVEMENTS (1987-88)

1) Work on stage 1 has almost been completed.
2) A number of suitable combinations of growth regulators have been formulated which provide good quality calli in reasonable quantity.
3) It has been established that with proper concentration and combinations of growth regulators and with serial subculturing of the explant, the "browning" problem can be minimized if not completely eradicated.
4) Physical conditions for proper experimentation have been established.
5) Surface sterilization procedures have been established.
6) Calli have been obtained through mesocarp tissue.
7) Work on stage No.2 regarding the initiation of suspension culture, has been initiated and atleast 3 batches are getting along very well. There is a definite increase in cell density.

CONSTRAINTS

So far, the major constraint has been the delay in the arrival of laboratory instruments. This has directly resulted in the loss of labour and time (e.g. results of many batches are not available because they were destroyed by contamination). This single factor is also responsible for slight lagging in achieving the goals set for the 1st year.

TABLE-10 EFFECTS OF GROWTH REGULATORS ON THE QUANTITY AND QUALITY OF Calli FORMED USING FULL STRENGTH MS MEDIUM

Batch No.	Plant Growth Regulators (Mg/l)									Callus	
	2,4-D	IAA	NAA	IBA	KIN	BAP	2iP	GA_3	Other GR if any	Quantity	Friability
1	2	3	4	5	6	7	8	9	10	11	12
15	1									++	Fairly friable
28	1.6									++	Compact
29	2.4									-	-
30	3.2									+++	Friable
31	4									+	Compact
32	3.2									+++	Fairly friable
33	4									+++	Friable
35	4.8									-	-
36	5									-	-
37	6									-	-
38	7									-	-
39	5.33									-	-
40		1								-	-
41		2								-	-
42		3								-	-
43		4								+	-
50		0.5								-	-
51		0.6								-	-
52			1							+	Fairly friable
53		2								++	Friable

TABLE-10 (Continued)

1	2	3	4	5	6	7	8	9	10	11	12
23	0.2			5		5		0.5		+++	Fairly friable
24	3				3					-	-
25	5				5					-	-
26	4				2					-	-
27	5				1					-	-
69	1	2			1					++	Compact
71	2			2	2					+	Compact
72	3		3		3					+++	Friable
73	1		1		1					+++	Fairly friable
74	2	2			2					++	Friable
76	4		2		2					++	Compact
77	4		4		4			0.5		+++	Fairly friable
80	2			5	2	5		0.5		+++	Friable
82	2	2			2			0.5		+++	Compact
83	3	3			3			0.5		++++	Friable
84	2		2		2					+++	Fairly friable
85	1.5	1	1		2					-	-
87	1	2			1				Casein hydrolysate 1 %	+++	Friable
88	2				2				Yeast extract 1 %	-	-
69		10×10^{-6} M				5×10^{-6}M				-	-
44						1				-	-
45						2				-	-
46						3				+	Compact
47						4				-	-
48				5						-	-

TABLE-10 (Continued)

1	2	3	4	5	6	7	8	9	10	11	12
54			3							+	-
55			4							-	-
56			5							++++	Highly friable
57		5								-	-
58		6								-	-
59				1						-	-
60				2						-	-
61				3						-	-
62				4						+	Fairly friable
63				5						-	-
64				6						-	-
70	10^{-5}M									+++	++
78	10									+	-
1			1			1					
2			3			0.5				+	Compact
3			5			0.5				-	-
4			4			1				-	-
5			3			1				++	Fairly friable
8			2			1				-	-
9	2				2					++++	Highly friable
16	2		2		2					++	Compact
17	0.5	2			0.5					++++	Highly friable
19	3				2					+++	Friable
21		2							Adenine	-	-

TABLE-11: EFFECTS OF GROWTH REGULATORS AND HALF STRENGTH MS MEDIUM ON THE QUALITY AND QUANTITY OF Calli FORMED

Batch No.	Plant Growth Regulators (Mg/l)									Callus	
	2,4-D	NAA	IAA	IBA	KIN	BAP	2iP	GA_3	Other GR if any	Quantity	Friability
1	2	3	4	5	6	7	8	9	10	11	12
112	2				2					-	-
86	2				2					++++	Highly friable
113	3				2					++	Friable
114	1									-	-
115		5								-	-
116	0.5		2		0.5					-	-
117	0.2			5		5		0.5		++	Friable
118	2	2			2					-	-
119	4									++	Friable
120	3.2									++	Friable
121	4	2			2					-	-
122	2		2		2					++	Friable
123	3		3		3			0.5		+	Fairly friable
124	1		2		1					+	Fairly friable
125	1	1			1					+	Fairly friable

TABLE-12: EFFECTS OF GROWTH REGULATORS ON QUANTITY AND QUALITY OF Calli FORMED WHEN 1% SUCROSE AND FULL STRENGTH MS MEDIUM WAS EMPLOYED

Batch No.	Plant Growth Regulators (Mg/l)									Callus	
	2,4-D	IAA	NAA	IBA	KIN	BAP	2iP	GA_3	Other GR if any	Quantity	Friability
1	2	3	4	5	6	7	8	9	10	11	12
132										-	-
133	10^{-5}M									+	Fairly friable
134	2				2					+	Compact
135	4				2					+	Compact
136			5							-	-
137			1			1				-	-
138			2			2				-	-
139	1		1		1					-	-
140	2		2		2					-	-

TABLE-13: EFFECTS OF GROWTH REGULATORS ON THE QUANTITY AND QUALITY OF Calli FORMED USING WOODY PLANT MEDIUM IN FULL STRENGTH

Batch No.	Plant Growth Regulators (Mg/l)									Callus	
	2,4-D	NAA	IAA	IBA	KIN	2iP	BAP	GA_3	Other GR if any	Quantity	Friability
1	2	3	4	5	6	7	8	9	10	11	12
90	2				2					+++	Friable
91	1									+	Compact
92			2							+	Compact
93	4									++++	Friable
94	3.2									-	-
95	0.5		2		0.5					-	-
96	0.2			5			5	0.5		+++	Compact
97	2	2			2					-	-
98&99	4	2			2					++	Friable
100										-	-
101	3		3		3			0.5		+++	Friable
102			10×10^{-6}M				5×10^{-6}M			-	-

TABLE-14: EFFECTS OF GROWTH REGULATORS ON THE QUANTITY AND QUALITY OF Calli FORMED USING ½ STRENGTH WPM

Batch No.	Plant Growth Regulators (Mg/l)										Callus	
	2,4-D	NAA	IAA	IBA	BAP	Kinetin	2iP	Zeatin	GA_3	Other GR if any	Quantity	Quality
1	2	3	4	5	6	7	8	9	10	11	12	13
103A	2					2					+	Compact
102			10×10^{-6} M		5×10^{-6} M						+	Compact
103B	3					2					++	Compact
103C	4	2				2					+	Compact
103D		5									++	Fairly friable
103E	3	3				3					+	Compact
103F	0.2				5	5			0.5		++	Fairly friable

TABLE-15: INDUCTION OF CALLUS FROM EXPLANTS

atch No.	Auxins (mg/l)	Cytokinins (mg/l)	Salt mixture	Callus induced from
1	2	3	4	5
30	2,4-D(3.2)		MS(full strength)	Nucellar tissue (?)
9	2,4-D(2)	Kinetin(2)	"	Cotyleon
17	2,4-D(0.5) IAA(2)	Kinetin	"	Cotyledon
19	2,4-D(3)	Kinetin(2)	"	Cotyledon
83	2,4-D(3) IAA (3)	Kinetin(3)	"	Cotyledon
56	NAA(5)		"	Cotyledon
72	2,4-D(3) NAA(3)	Kinetin(3)	"	Cotyledon
36	2,4-D(2)	Kinetin(2)	MS(half strength)	Cotyledon
23	2,4-D(0.2) IBA(5)	BAP(5) + GA_3(0.5)	MS(full strength)	Cotyledon
13	2,4-D(4)		WPM(full strength)	Cotyledon
19	2,4-D(4) NAA(2)	Kinetin(2)	"	Cotyledon
11	2,4-D(3) IAA(3)	Kinetin(3)+ GA_3(0.5)	"	Cotyledon
9	2,4-D(4)		MS(half strength)	Cotyledon
0	2,4-D(2) IBA-(5)	BAP-(5) Kinetin(2)	MS(full strength)	Seed coat
6	2,4-D(2)	Kinetin(2)	MS(half strength)	Seed coat
3	2,4-D-(1) NAA-(1)	Kinetin(1)	MS(full strength)	Seed coat
0	2,4-D(3.2)		"	Seed coat

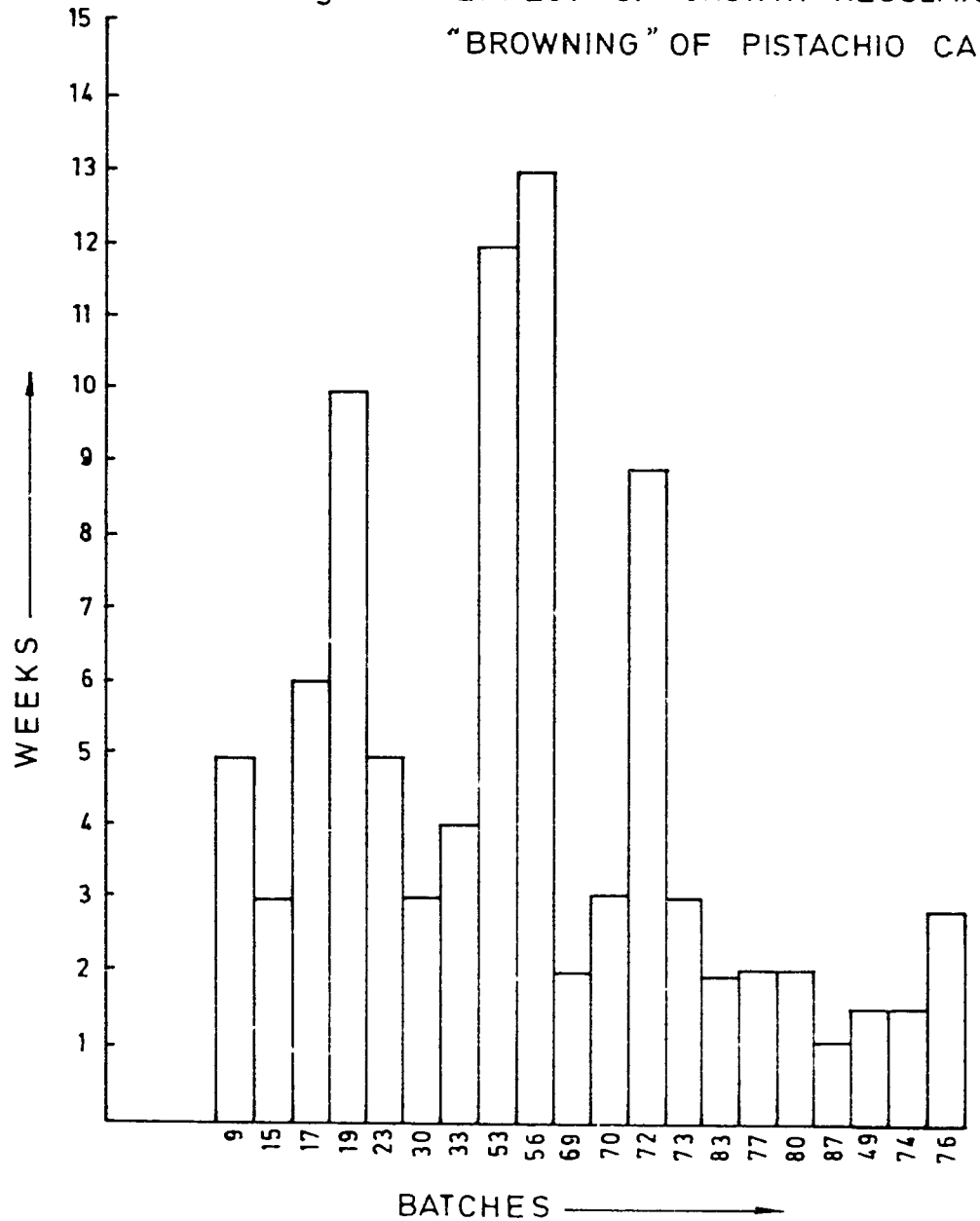
TABLE-15(Continued)

1	2	3	4	5
119	2,4-D(4)		WPM(full strength)	Seed coat
15	2,4-D(1)		MS(full strength)	Embryonic axis
73	2,4-D-(1) NAA(1)	Kinetin(1)	"	Embryonic axis
89	IAA (10×10^{-6}M)	BA(5×10^{-6}M)	"	Embryonic axis
92	IAA(2)		WPM(full strength)	Embryonic axis
87	2,4-D-(1) IAA-(2)		MS(full strength)	Embryonic axis
12	NAA(1)	BAP(1)	"	Shoot
137	NAA(1)	BAP(1)	"	Shoot
147	2,4-D(3)	Kinetin(2)	"	Mesocarp
148	2,4-D(4) NAA(2)	Kinetin(2)	"	Mesocarp

TABLE-16: INITIATION OF SUSPENSION CULTURE

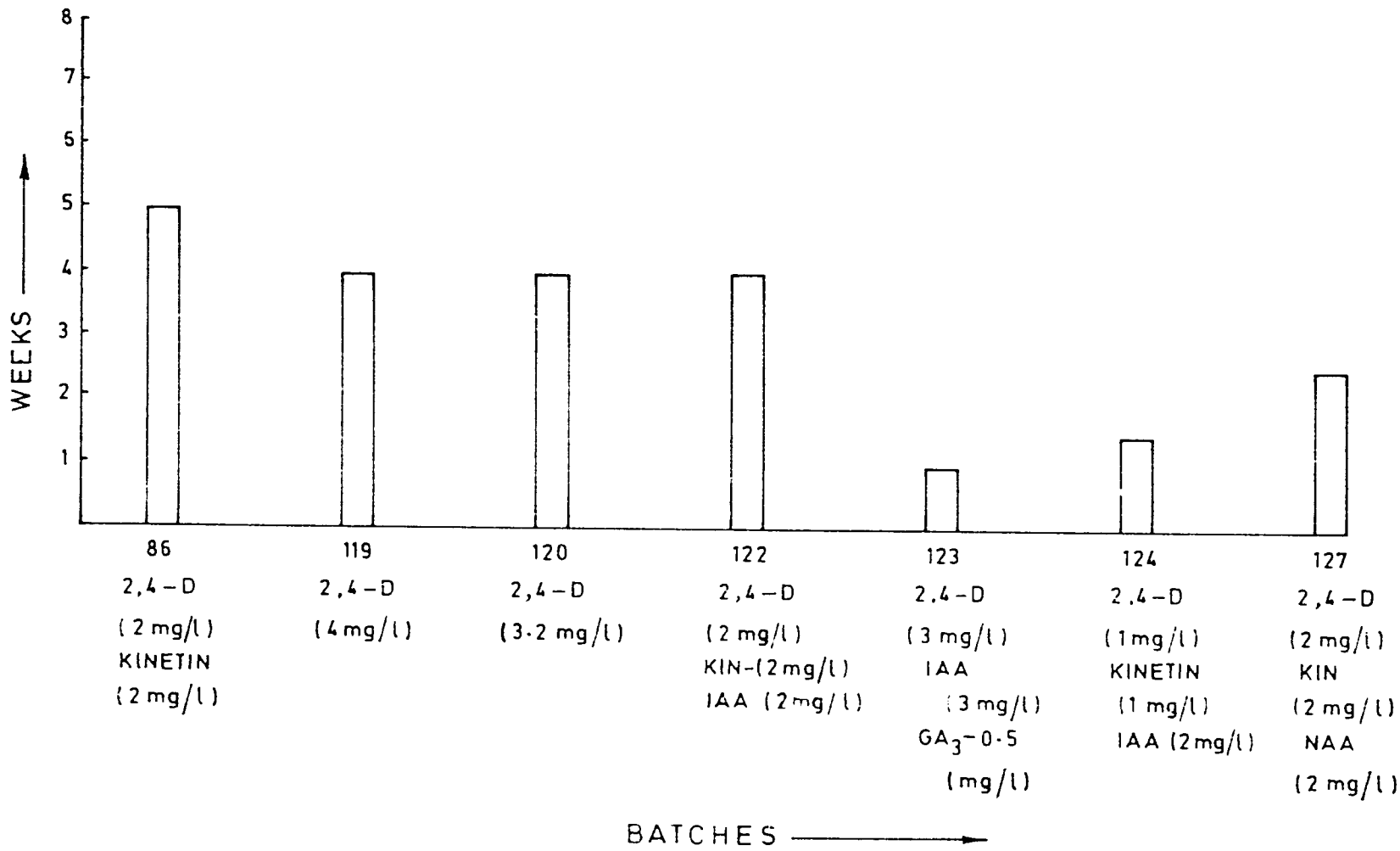
Batch No.	Inoculum obtained from	Auxin (mg/l)	Cytokin (mg/l)	Inoculum density in cell per ml	Remarks
151	151(9)	2,4-D(2)	Kinetin(2)	$2x10^4$	Batch destroyed due to contamination
152	178	2,4-D-(0.5) IAA-(2)	Kinetin(0.5)	$1x10^4$	Batch getting along very well(results awaited
153	164	2,4-D-(3) IAA-(3)	Kinetin(3) also GA_3 (0.5)		Results awaited
154	159	2,4-D-(0.2)	BAP (5) also GA_3 -(0.5)		Results awaited
155	156	2,4-D-(3) NAA-(3)	Kinetin(3)		Destroyed by contamination
156	157			$4x10^4$	Results awaited
157	153	2,4-D-(1) NAA-(1)	Kinetin(1)	$1x10^4$	Results awaited
158	166	2,4-D(3)	Kinetin(2)	$4x10^4$	Results awaited

Fig. 1. EFFECT OF GROWTH REGULATORS ON "BROWNING" OF PISTACHIO CALLI *



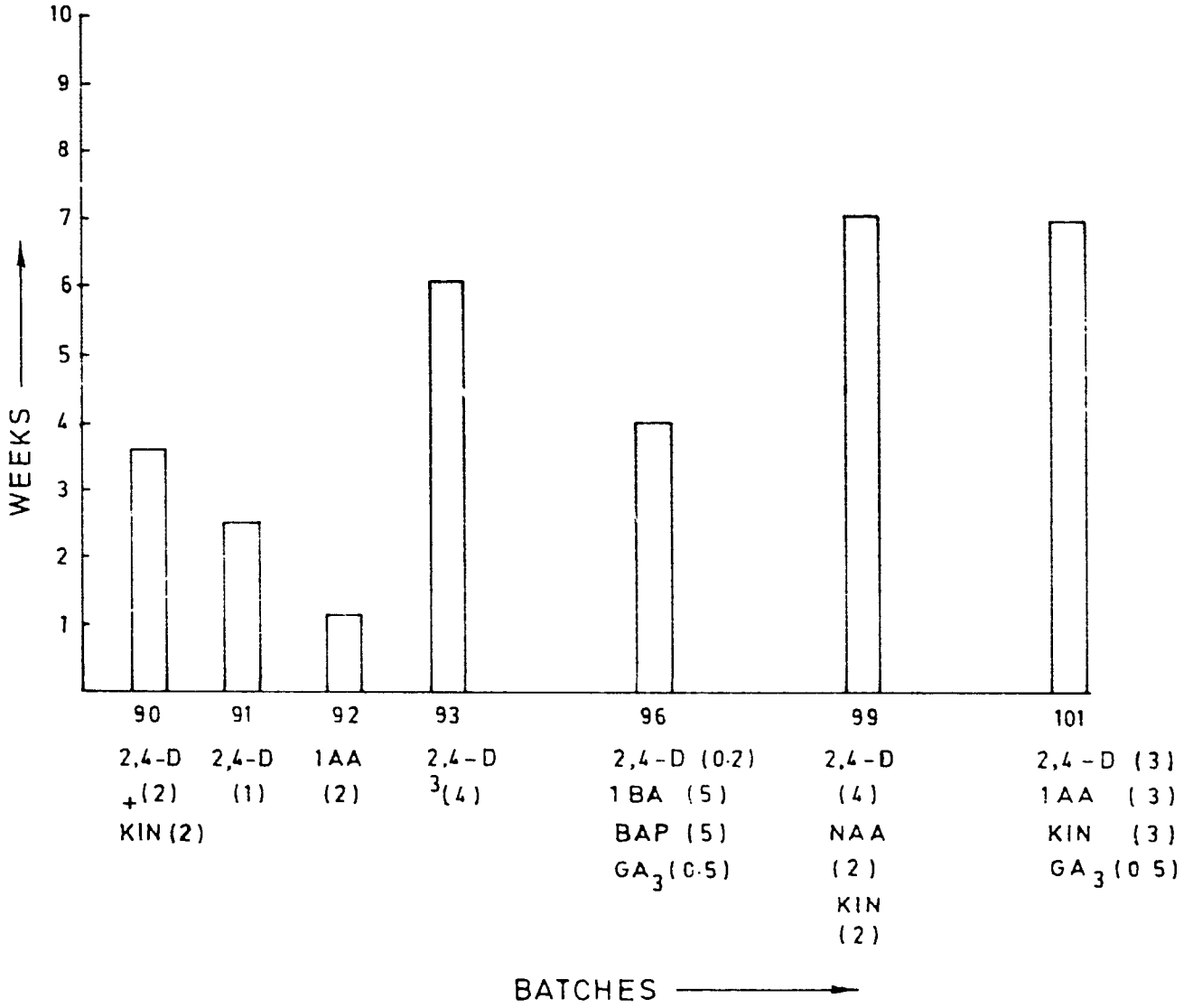
* FULL STRENGTH M.S. MEDIUM

Fig. 2. EFFECT OF GROWTH REGULATORS ON "BROWNING" OF PISTACHIO CALLI *



* HALF STRENGTH MS MEDIUM

Fig. 3 EFFECT OF GROWTH REGULATORS ON "BROWNING" OF PISTACHIO CALLI *



ANATOMICAL STUDIES OF IN VITRO CULTURED P. VERA EXPLANTS

Histological examination of hormone induced dedifferentiation and redifferentiation of *in vitro* cultured *P. vera* explants and their comparison with *in vivo* differentiated tissue helps to conclude specific mode of action of any hormone related to ultra structural/morphological changes.

Plan of Work

1. Study of histological features of shoot and root tips of seedlings cultured on hormone free medium.
2. Analysis of hormone induced anatomical changes in calli with/without any morphological changes leading to organogenesis or embryogenesis.
3. Comparison of calli propagated on different media.
4. Histological examination of competable and non-competable micrograft union sections.

Histological changes in the sections of explants, which included cotyledons, eoi, meso and hypocotyles and tips of the roots, shoots and seedling, grown on MS medium with or without supplementation with hormones were studied and recorded in the form of photographs.

Microtechnical procedures leading to microscopy included fixation of explants in FAA (Formaline, Acetic acid and alcohol) mixture. The explants were dehydrated by passing through different concentrations of TBA (tertiary butyl alcohol), absolute alcohol

and water. Finally the sections were prepared by microtomy of the paraffin embedded explants. Free hand cutting and smear methods were also applied for calli and globular embryoids.

Delafield hematoxylin staining method was adopted to locate zones formed with aggregates of cells densely filled with cytoplasm. Safranin-fast green staining procedure was suitable for detection of induced tracheal element in the calli.

Some photographic results of different hormonal combinations on callogenesis of cotyledonary explants of P. vera are depicted in photographs. Full strength MS medium supplemented with the following concentrations of growth regulators was employed:

2,4,D	2.0 mg/l
Kinetin	2.0 mg/l
IBA	2.0 mg/l
Yeast ext.	0.5 % w/v

Photograph Nos.22,24,48 and 50 show abnormal swelling on the mesocotyle portion when cotyledons were cultured on MS medium supplemented with hormones.

On the peripheral part of the cortex region and below the hypodermus darkly stained, compact group of cells were developed, which were different in shape from the normal cortex cells. These were small rounded cells, without intercellular space between, and were densely filled with cytoplasm. This type of cells was also present within the combium cells. Endodermis and pericycle showed abnormal growth and xylem and phloem region were well differentiated. Outside the pith region large number of small

size, dark, tightly packed cells were present. Pith cells were larger in size than the surrounding cells.

Callogenesis from cotyledons propagated on full strength MS medium containing 2,4,D, 0.2 mg/l, Kinetin 2.0 mg/l, 2iP 2.0 mg/l, Sucrose 3.0 % is depicted in photograph Nos.18 (20), 44 (46),76 and (78). Two types of cells-one of irregular shape and the other round, were present. Dark region of the photographs indicated presence of dense cytoplasm in the cell.

Photograph Nos.28,72 (74),52 and (54) were from the cotyledonary callus grown on MS medium with NAA 1.0 mg/l, NH_4Cl 100.0 mg/l, Sucrose, 3.0 % and BAP 2.0 mg. Two types of cells some round in shape and similar to cotyledonary cells, others elongated were seen. These cells were darkly stained and showed dense cytoplasmic contents.

Photograph Nos.80 and (84) showed callus formation from cotyledon due to the influence of NAA 4.0 mg/l and sucrose 3.0 %.

Callus cells were irregular in shape. Some were small in size and others large at 25X magnification. These cells were well stained and were different from normal cotyledonary cells. Difference in shape and size demonstrated effect of NAA. Dark staining showed presence of cytoplasm,

Photograph Nos. 30,32 (34),38 and 40 show callogenesis from cotyledon and its morphogenic changes due to the effect of 2,4-D (4 mg/l). Two types of cells were present in photograph No.30. Some were round in shape and darkly stained, others were

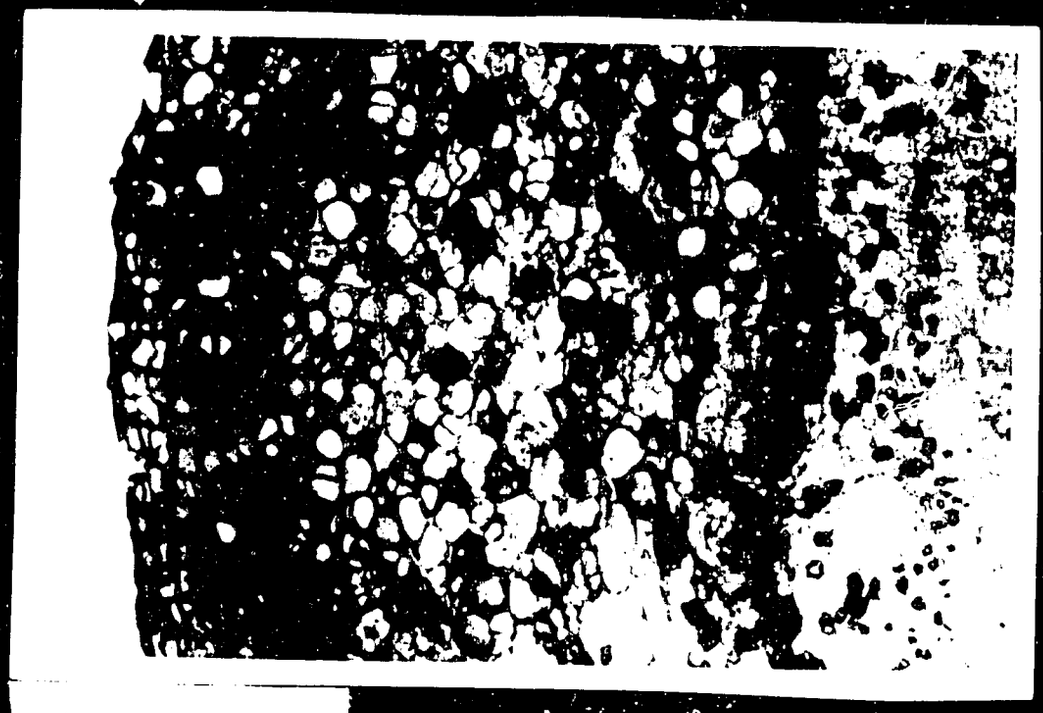
groups of conducting strands. There strands showed trachery elements formation in the callus. Photograph Nos.32,34 and 38 showed rounded cells, whereas irregulars, rounded and elongated cells arranged in two rows, were present in photograph No.40. This variety of cells showed induction of organogenesis in the callus and dedifferentiation of cells.

Photograph Nos.56 (58),60.(62),68 and (70) showed callogenesis on supplementation with auxin and cytokinin,2,4-D 1.0 mg/l, Kinetin 1.0 mg/l and NAA 1.0 mg/l. Elongated cells, irregular cells and rounded cells are present in these photographs. The cells are tightly packed and no intercellular space is present between them. All these cells were darkly stained, filled with cytoplasmic contents and small vacuoles were present in these cells.

Root regeneration from cotyledon in the presence of kinetin (2.0 mg/l) and 2,4-D (2.0 mg/l) is depicted in photograph Nos.26,64 (66),which show well differentiated reactions of trachery elements. Double walled xylem cells were also clearly visible. Paricycle cells around the phloem tissues were well differentiated and darkly stained. Two types of phloem meta and proto-phloem were clear. Pith cells were large in size and darkly stained. Dark staining of pith cells, pericycle and cambium showed that they were densely filled with cytoplasm

DISCUSSION

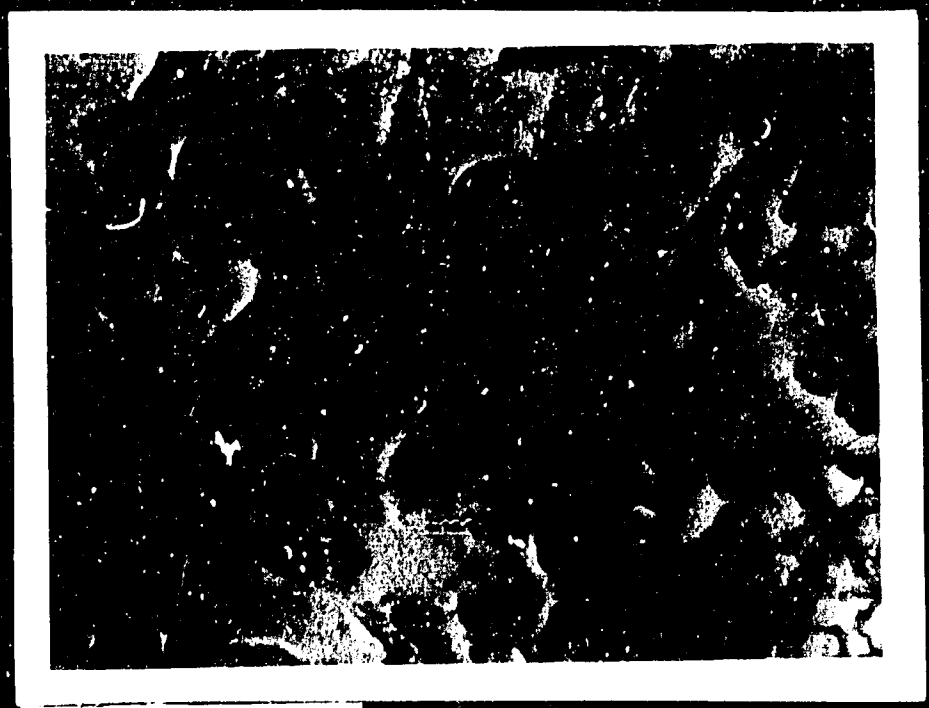
Root formation was invariably induced by 2,4-D from cotyledonary callus and mesocotyledonary formation. Sections with 2,4-D and kinetin showed well differientiated trachery elements. Xylem elements were greater than phloem elements. Pericycle and combium region showed abnormal growth. Increase in concentration of 2,4-D from 2-4.0 mg/l in the medium resulted in the formation of conducting strands. These strands were similar to the well developed trachery elements of root and mesocotyle portion. Addition of hormones initiated calloqenesis due to which elongated and irregular cells and round cells were formed. These cells were tightly packed, without intercellular spaces and full of cytoplasmic contents. Concentration of NAA (1.0 mg to 4.0 mg/l) in the medium showed calloqenesis from cotyledons in which elongated or cylindrical cells and irregular cells were formed. They were darkly stained which showed presence of dense cytoplasm. These cells were entirely different from the simple cotyledonary cells.



PHOTOGRAPH NO.22

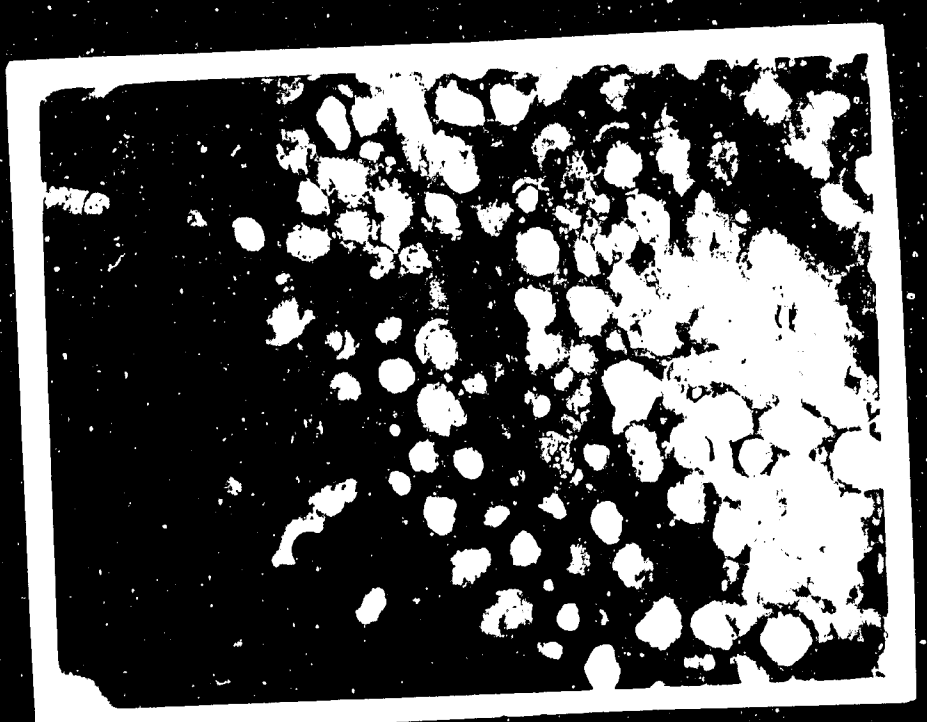
PHOTOGRAPH NO.24

PHOTOGRAPH NO.44

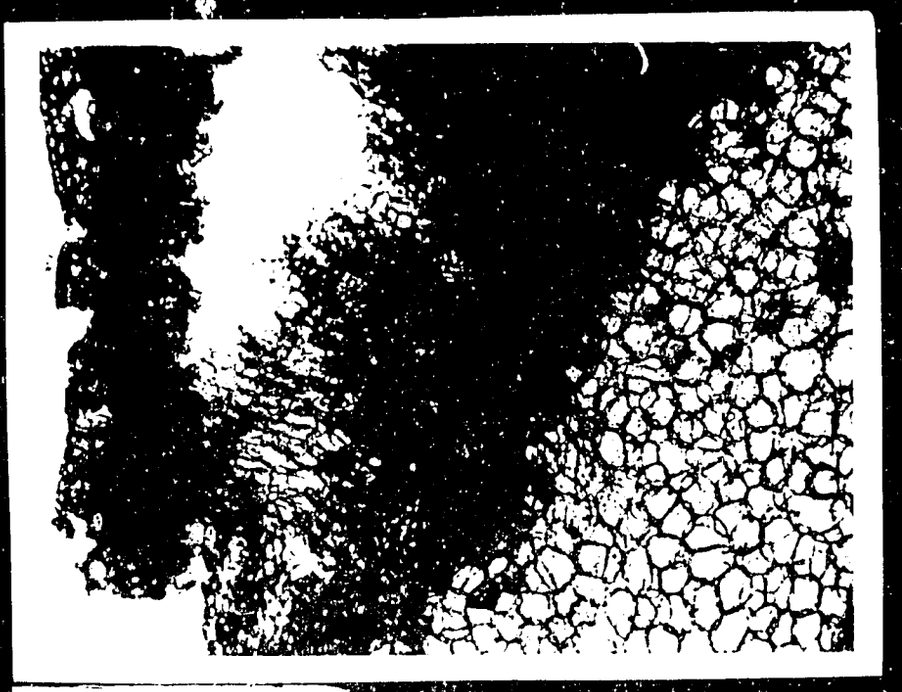


PHOTOGRAPH NO.28

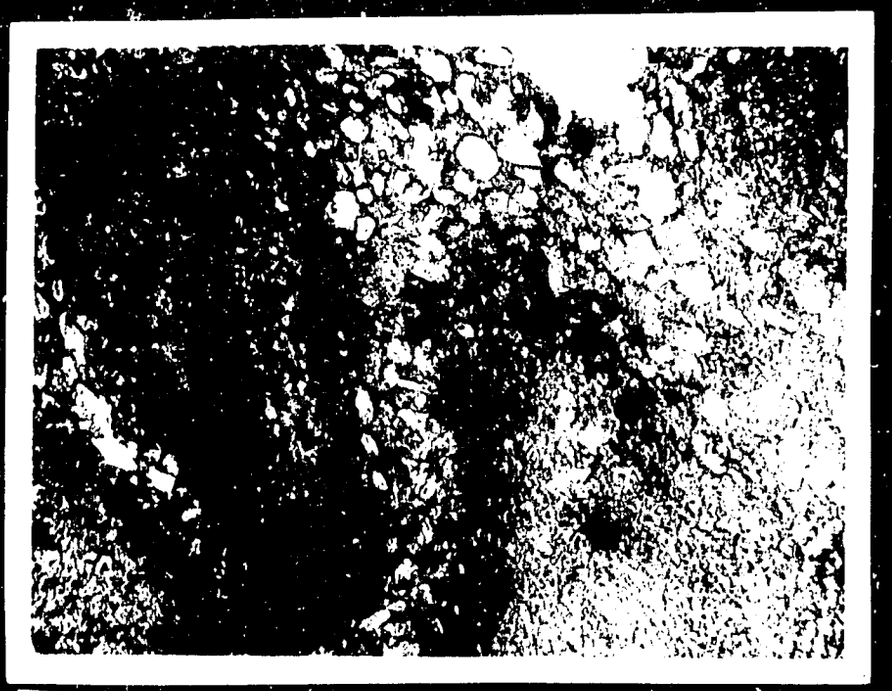
PHOTOGRAPH NO.68



PHOTOGRAPH NO.26



PHOTOGRAPH NO.52



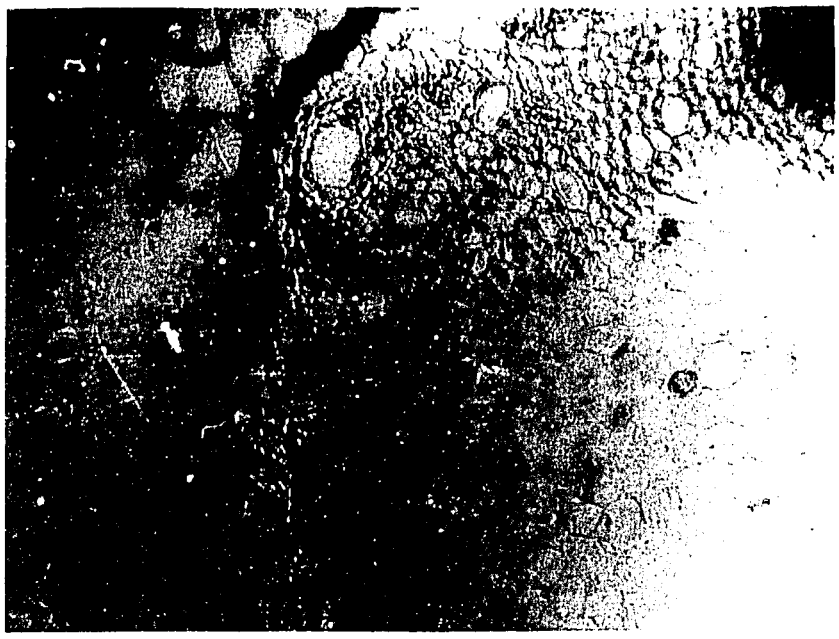
PHOTOGRAPH NO.80

PHOTOGRAPH NO. 38

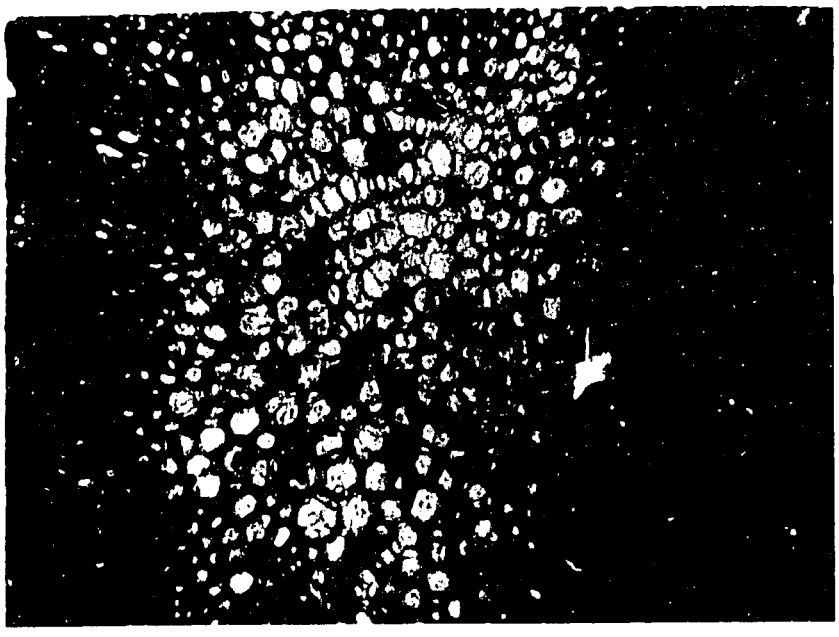
PHOTOGRAPH NO. 40

PHOTOGRAPH NO.56

PHOTOGRAPH NO.60



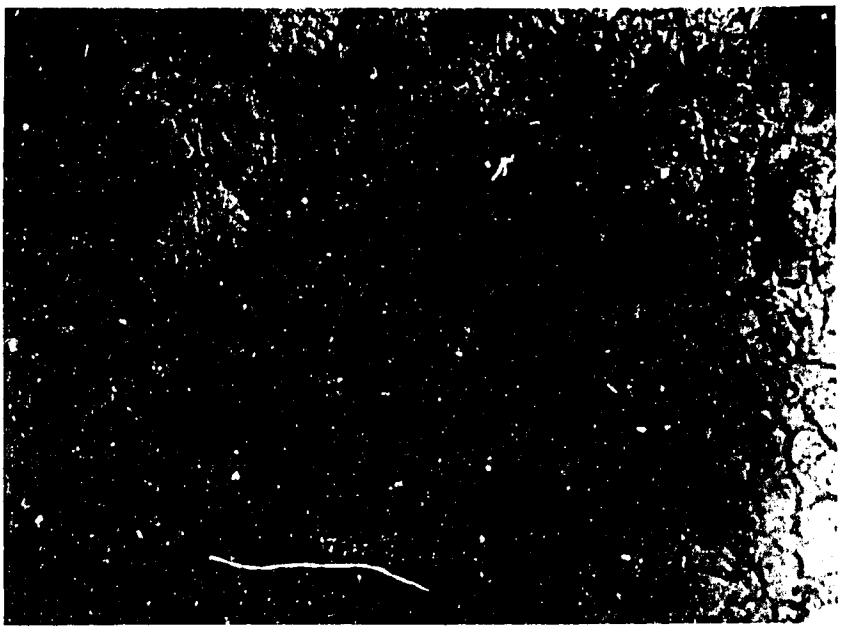
PHOTOGRAPH NO.64



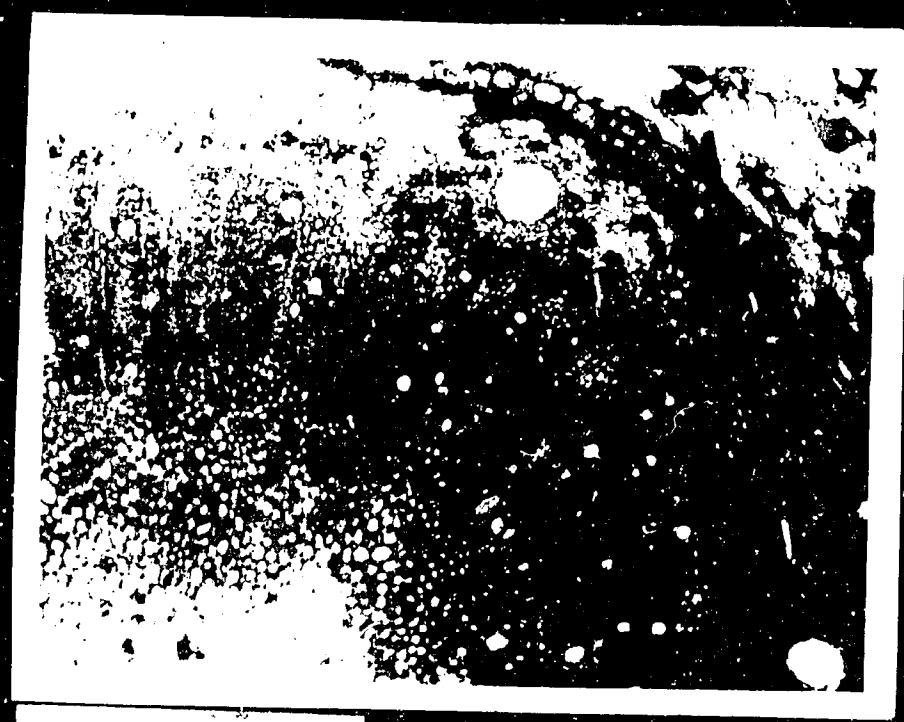
PHOTOGRAPH NO.50

PHOTOGRAPH NO.30

PHOTOGRAPH NO.32



PHOTOGRAPH NO.76



PHOTOGRAPH NO.48

PHOTOGRAPH NO.18

PHOTOGRAPHIC ADDENDUM

Photographs showing typical results of:-

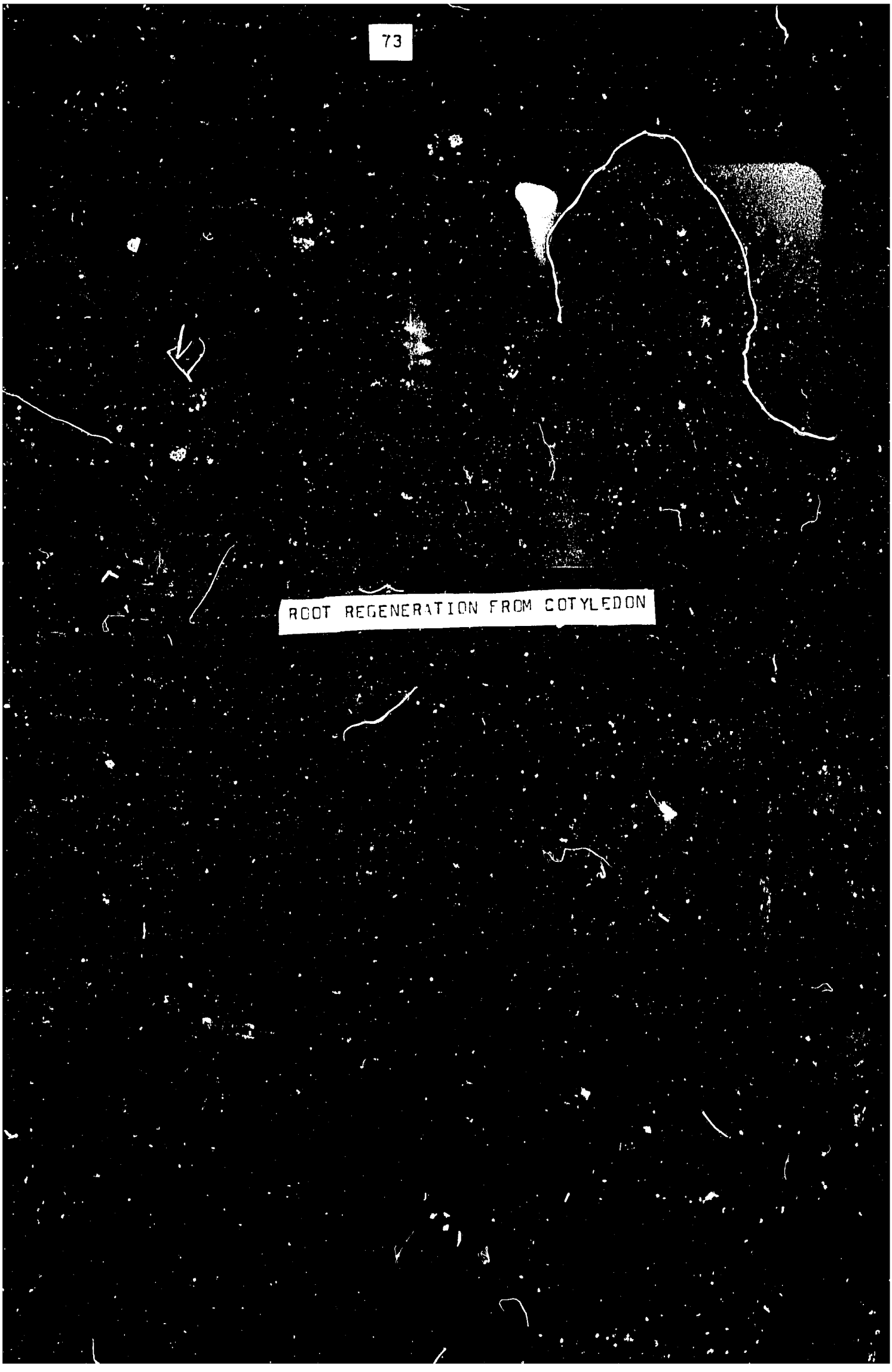
i) Growth of seedlings

ii) Regeneration of roots from cotyledon

iii) Induction of callus from cotyledonary explant

SEEDLING GROWING IN THE PRESCENCE OF GROWTH REGULATORS

SEEDLING GROWING IN THE BASAL MEDIUM



ROOT REGENERATION FROM COTYLEDON

COMPACT CALLUS FROM COTYLEDON

FRIABLE CALLUS FROM COTYLEDON

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 composition 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 scientists 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).

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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 <u>in vitro</u>, 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. <u>Pistachio nut</u> 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,

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(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 shoots 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); Gamborg (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.

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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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LAHORE

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 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 transformation 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.

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 propagatio *in vitro* as an invaluable aid to the cropping of trees appea to be assured.

The woody species that have been successfully propagat 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 Jaran[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 develope by various workers to suit particular requirements of 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.

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CLONAL PROPAGATION OF PISTACHIO THROUGH TISSUE CULTURE

SYNOPSIS FOR DOCTORAL WORK

IN

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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 the *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,[9] (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.

FIFTH 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.

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