Effect of Phytohormones in the growth of Pomegranate (Punica granatum) in In- Vitro Condition

A
THESIS
SUBMITTED TO THE

KIIT SCHOOL OF BIOTECHNOLOGY,
KIIT UNIVERSITY BHUBANESWAR
IN PARTIAL FULFILLMENT OF THE REQIUREMENTS FOR THE AWARD OF THE DEGREE

OF
MASTER OF SCIENCE

IN

BIOTECHNOLOGY

BY

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M.Sc (BIOTECH) May 2018

CERTIFICATE

This is to certified that the thesis entitled " The effect of Phytohormones on the growth of Pomegranate (Punica granatum) in In vitro condition submitted by Ms. Sangya Sharma in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology" of the **KIIT SCHOOL OF BIOTECHNOLOGY** is a record of bonafide research work carried out by her under my personal guidance and supervision.

Place: Ahmedabad **(Dr. Shishir Shrivastava)**
Date: Research Guide

ACKNOWLEDGEMENT

The work presented in this dissertation was performed in Tissue Culture Laboratory, Cadila Pharmaceuticals Limited, at Hirapur 2018. I would like to thank **Dr. Kiran Ghanti AGM** for his continuous support during this time and for providing such an exciting and collaborative environment to work.

I would like to thank **Senior Manager- Human Resource Mr. Mehul Vaghela** for providing such a platform to leverage my knowledge and career.

There are many people without whom I would not have reached this point. I would like to begin by thanking **Dr. Avishek Nath DGM(R&D head),** I would like to thank **Dr. Shishir Shrivastva** their passion for science is truly inspiring, and your unwavering support has taken me to great heights, and for that I am forever indebted to you .

You have been an instrumental part of my scientific development, and the lesson I have learned during my time I your lab will go with me throughout my career

I would like to thank **Mr. Bhavin Patel and Mrs. Reeshma Shah** for their divine support in learning all the techniques and management in Production.

I would like to thank **Mr. Hasmukhbhai Solanki** for teaching me all the basics of chemicals and media preperation.

Above all the people mentioned above there are few people without whom I won't be able to complete my work thanks to the team of R&D**(Amarsingh Gohil AG, Vinu Baraiya VG, Bhim Singh BH)** for teaching me all the basics of tissue culture.

Finally, I would like to thank my parents **Dr. Hari Om Sharma and Mrs. Meenakshi Sharma** and my all time support system my elder brother **Sidharth Sharma**. Without them I would not be able to reach till this phase of my life. Education has always been the highest priority in my family, and I am eternally grateful to my parents for imparting their positive values in my life.

Though my experiences in Cadila Pharmaceuticals, I have discovered one thing that **"You doesn't need anything extraordinary to achieve success. Timely decisions are the key."** I am thankful to everyone who has helped me to reach this point.

Date: 8.05.2018 Sangya Sharma
Place: Ahmedabad

LIST OF TABLES

LIST OF ABBERVIATIONS

Anon	**Anonymous**
cv	Cultivar
℃	Degree Celsius
et.al	and others
gm	gram
IAA	Indole -3- Acetic Acid
IPA	Isopropyl alchol
Kg	Kilogram
Kn	Kinetin
Mean	Arithmetic mean
mg	Milligram
M	Micron
mg/lit	Miligram per liter
MS	Murashige and Skoog
NAA	Napthalene Acetic Acid
No	Number
%	Percent
psi	Pound per square inches
RO	Reverse osmosis water
RPM	Rotation per minute
S.D	Standard deviation
S.Em	Standard error of mean

UV Light	Ultraviolet light
w/v	Weight/ Volume

ABSTRACT

Punica granatum (Pomegranate) is a fruit bearing deciduous shrub or small tree in the family Lythraceae that grows between 5 and 8m tall. Fruit is also a good source of many vital B- complex groups of vitamins such as pantothenic acid, folates,pyridoxine, copper, potassium, manganese. In Vitro micropropagation of Pomegranate was tried using axillary bud, to produce true to type, disease free plants having known sex. For growth of explant different hormones are used IBA,IAA, BAP,NAA, AgNO3, ADS in order to solve mainly two problems in Pomegranate.

1. To increase no of shoots
2. Removal of Callus

Following criterias are examined

Percentage of sprouting, Percentage of shoot induction, Average number of leaves, Average length of shoots, Percentage of root induction , Average number of roots, Average root length, Callus Growth.

The effectiveness of BAP,NAA, AgNO3 was proved superior to auxin with respect to percentage of sprouting (98.41) in this (MS+ BAP 1.8mg/lit+NAA

0.9mg/lit+AgNO3 1mg/lit+ADS 30mg/lit), the highest shoot length(4.92cm)

For Rooting media used is MS+ IAA,IBA,NAA.The highest percentage of root induction (88%) were found in MS+IAA(0.1mg/lit)+NAA(0.125mg/lit). After 28 days of culture, the lowest value for root characters like percentage root induction and number of root per shoot was recorded. BAP shows adverse effect on root growth and development in Pomegranate.

Moreover, addition of auxin (IAA at 1.25 mg/lit) induced adventitious rooting. The combination of both the hormones did not give the highest count for any character under study . For establishment and growth of Pomegranate axillary explant MS basal media supplied with MS+ BAP 1.8mg/lit+NAA 0.9mg/lit+AgNO3 1mg/lit+ADS 30mg/lit) was the best media composition.

CHAPTER 1

INTRODUCTION

GENERAL INFORMATION

The pomegranate (Punica granatum) is a fruit bearing deciduous shrub or small tree in the family Lythraceae that grows between 5 and 8m(16 and 26ft) tall.
Completely established tree bears many round, bright red, purple or orange-yellow coloured fruits depending on the cultivators types.
An average each fruit measures 6-10 cm in diameter and weight about 200 gms. Its outer skin is tough features leathery textures and interior structure of the fruit is seperated by white, tiny, spongy, membranous, bitter tissue into discrete compartments.
Such sections packed as sac, filled with tiny edible sweet juicy, pink ails encasing around a single, angular, soft or hard seed.

SCIENTIFIC CLASSIFICATION

KINGDOM: Plantae
DIVISION: Magnoliophyta
CLASS : Magnoliopsida
ORDER : Myrtales
FAMILY: Lythraceae

GENUS: Punica
Species: P. granatum.

NUTRITIONAL VALUE

Pomogranate carries about 83 calories per 100 grams. It contains no cholestrol or saturated fats. It is a good sourse of soluble and insoluble dietary fibres; providing 4 grams per 100 grams.

Certain ellagitannin compounds such as GranatinB and Punicalagin are found abundantly in the pomegranate juice.

Fruit is a good source of antioxidant vitamin c. Antioxidant strength of pomegranate fruit measured regarding its Oxygen Radical Absorbance Capacity.(ORAC) is 2341micromolTE/100g.

Fruit is also a good source of many vitalB- complex groups of vitamins such as pantothenic acid, folates,pyridoxine, copper, potassium, manganese**.**

AREA AND DISTRIBUTION

POMOGRANATE PRODUCING STATES IN INDIA

STATE	AREA (hectare)	PRODUCTION (tonnes/hectare)
ANDHRA PRADESH	5.6	56.4
CHHATISGARH	0.1	0.4
GUJARAT	4.4	45.6
HIMACHAL PRADESH	1.3	0.5
MAHARASHTRA	98.9	555.5
KARNATAKA	13.2	138.5
NAGALAND	0.1	0.3
ORRISA	0.2	0.8
TOTAL	123.8	798

(NHB 2016)

WHY TISSUE CULTURE IN POMOGRANATE

Traditionally pomegranate is multiplied through stem cutting. Propagation through seeds is not desirable resulting cross pollination, maintenance of true to type plant is another major problem.

Stem and root cuttings are labour intensive and also requires bulk amount of veins and roots, which restrict their multiplication at commercial level.

In vitro multiplication of elite clones will be an attractive approach in order to meet the requirements of quality propagules at large scale for commercial cultivation.

The present study was therefore undertaken to develop a rapid micropropagation protocol for plant regeneration of pomegranate through In vitro culture of shoot tip and nodal segment as reported by **Malek et al . (2007).**

HISTORY OF PLANT TISSUE CULTURE

This ability has been known for many years and much information has been gathered on the best way to look after plant materials of different species and sources for Different uses using plant tissue culture. **Vochtung** in **1878** observed that cells along a stems length were capable of generating roots or shoots. **Gottlieb Haberlandt** a german botnist was the first to generate tissue from fully differentiated tissue. He was interested in this as he thought it would give.

Interesting insight into the properties and potentially which the cell as an elementary organism process.

Hanning grew nearly mature embryos of crucifers and grew them to maturity in mineral salts and sugar solution.The embryos will not grow to form plants however without the addition of growth compounds. Later work was successfully reared embryos that were otherwise unviable using tissue culture. Several new hybrids have since **evilved** using tissue culture that would otherwise have been unviable at the embryo stage.

Cell division in tissue had to await the discovery of **Kinetin.** This was first discovered by autoclaving freshly isolated slurries of DNA from Herring

sperm. The discovery of cytokinins gave much impetus to tissue culture.

Hormonal control of tissue culture was achieved using the regulation of cytokinin , auxin ratio controlling root and shoot development in tobacco.

Growing in cytokinin rich media can reduce apical dominance leading to more shoots and to quicker regeneration of numbers. In Vitro fertilization made it possible to cross varieties unable to cross in nature. Haploid plants of tobacco were raised from pollen grains. A more recent development in tissue culture in protoplast culture.

In General Following are the Benefits of Plant Tissue Culture In Pomegranate.

1. High rate of establishment:

TC raised plants have high rate of establihment (90 to 98%) against conventional propagates (55 to 65%).

2. True to type and elite plants:

Plants produced by tissue culture technology have uniform genotypic make up , which ensure target production. Such plants are prepared from plants of farmer’s criteria viz., good quality and high yeild. These benefits can not be avail with conventional planting material.

3. Disease free planting material:

Cuttings of pomegranate are brought from the old orchards. The disease history of that orchards may not be available. Cuttings from such feild carry disease causing organism in new feild and affects the survival and performance of new sprouts. Tissue culture raised planting material is free from any such diseases so have better growth and survival rate. For fulfill the demand of such quality propagules experiments were designed to standardize surface sterilization and establishment of cultures of pomegranate using axillary buds of explant.

4.Minimum space requirement for multiplication of large number of plants.
5.High yeild potential.
6.Rapid multiplication and early harvesting
7.Uniform size and age of plants
8.Available through out the year
9.Better marketing
10.Faster and better distribution

CHAPTER 2

REVIEW OF LITERATURE

The available literature pertaining to in vitro studies viz surface sterilization of Explant and in vitro studies are reviewed and presented in this chapter.

SURFACE STERILIZATION

Proper surface sterilization of explants before inoculation is essential to avoid the external contaminants that will affect the multiplication of explants.

Nabi et al .(2002) sterilized pomegranate explant by washing under running tap water in plastic pot for 10 to 15 min. Later,plants were cut into pieces and washed with detergent powder and 1-2 drops of Tween20 for 10 min and then sterilized with 0.1% HgCl2 solutions again with 1- 2 drops of Tween-20 for 5min followed by three - four rinses in autoclaved distilled water to remove traces of HgCl2 under laminar airflow cabinet.

Malek et al (2007) used detergent for 15 mins followed by 7 mins treatment with 0.1% (v/v) mercuric chloride with a few drops of tween 20 for surface sterlization than rinsed 4-5 time with sterilized water inside the clean bench to remove all traces of HgCl2 of shoot tips and nodal segments.

Young vines of Pomegranate were collected and washed with 1.0% Cetrimide solution

Followed by surface sterlization with 0.1% HgCl2 for 5 mins than explant will rinsed 4-5 times with sterilized double distilled water as repeorted by **Kumar et al 2003.**

The surface sterilization of shoot tips was done with 1% Savlon for 10 mins with constant shaking than washed with 3-4 times with distilled water and was immersed in 0.1% mercuric chloride solution with shaking for 4 mins under laminar airflows followed by washing with sterilize distilled water**(Abdul-Awal et al, 2005).**

In Vitro Studies:

Explant from healthy plant of appropriate physiological stage is preliminary requirement for any tissue culture study to get uniform and optimum results.

Debergh and Zimmerman (1991) reported that herbaceous plants were highly responsive to BAP treatment and most of the robust well formed shoots suitable for further shoot proliferation.

Zaman et al (1992) demonstrated similar effects of BAP on shoot elongation in nodal segment culture of verbena spp.

George, (1993) reported increasing concentration of BAP upto 2.0 mg/lit the no of shoots increased and then decreased at very high BAP concentration (2.5mg/lit).

The highest frequency of shoot formation (78%) with 7.9 shoot per explant in MS supplemented with 23.0 mg/l BAP reported by **Islam et.al (1994)** in teasel gourd.

Lee and Thomas(1995) suceeded in obtaining multiple shoot proliferation and shoot formation from shoot tip and stem node of Cucurbita foctidissima in MS supplemented with 0.1-1.0 mg/lit NAA.

Hoque et.al.,(1995) found that a combination of 1.5mg/lit NAA was more suitable for adventitious multiple shoot formation in teasel gourd.

M.J. Alam et.al.,(1997) reported multiple shoot formation was 100% for nodal segments when cultured on MS containing 1.0 mg/lit Kn + NAA whila 90% explants produced shoots on MS containing 1.0mg/lit BA with average number of shoots per explant was 3.44 0.61

Hossain et al. (1997) observed best shoot elongation in MS supplemented with1.0 mg/lit BAP, 0.1 mg/lit NAA and 10 mg/lit adenine sulphate.

Hoque et al.(1998) reported that shoot formation was achieved in seeding explants of T.diocia on MS medium supplemented with 4.44 microM BAP.

Uddin (2000) reported with the same concentration of BAP and Kn (2.5 mg/lit), lower nodal segment produced maximum percent of shoot (91.66%) than the upper nodal segment. The percent of shoot induction was decreased at MS + 2.5mg/lit BAP.

Debnath et al (2000) found 92% multiple shoot regeneration from shoot tips when cultured on a medium containing MS+2 mg/lit BAP + 0.1mg/lit NAA and found the longest shoot of 8.6cm for shoot tips, cultured on MS media supplemented with 1.0 mg/lit BAP + 0.1mg/lit NAA + 10 mg/lit adenine sulphate (AS).

The shoot induction percentages(75.15%) in pomegranate was reported by **Thomas (1999) and Uddin (2000).**

The results opened declared that nodal segment was more suitable for shoot regeneration and multiplication and also maximum shoot elongation. These results were in agreement with the findings of **Debnath et al.(2000) and Uddin (2000).**

The highest number of multiple and tallest shoots were observed in MS medium fortified with 1.0 mg/lit BAP and 0.1mg/lit NAA reported by **Nabi et.al(2002)** in Momordica diocia.

Sikdar et al.(2003) observed similar results and they reported that performance of the nodal explants for multiple shoot regeneration was better.

Higher number of shoots per explants (5.78), number of nodes per shoot (6.11) and longest shoot(3.57cm) were observed on the medium containing 8.88 micro M BA reported by **Kumar et al.(2003).**

Abdul- Awal et al. (2005) reported the frequency of multiple shoot formation was 100% for shoot tips in MS medium containing BA 1.0 mg/lit and BA 1.0 mg/lit + NAA 0.2mg/lit. The best results were found when explants were cultured on MS medium supplanted with 1.0 mg/lit BA.

Malek et al.(2007) has compared various concentration of BAP found that MS + 2.0 mg/lit BAP showed higher percentage of shoot induction (93.86%), maximum number of shoots per explants (3.25) and length of longest shoot per explants (4.25cm) from nodal segment and it was 84.71%, 2.50 and 4.18 cm, respectively, for

shoot induction percentage , shoot number per explants in shoot tips in medium with the same concentration of BAP.

CHAPTER 3

MATERIALS AND METHODS

The present dissertation on Micropropagation studies in Pomegranate(Punica granatum) was conducted at Tissue Culture Laboratory, Cadila Pharmaceuticals limited, 755, Prakruti farm, village Hirapur, Ta. Dascroi, Dist, Ahmedabad- 382435 during January- May (2018).

Brief description of procedures used for initiation of aseptic cultures, multiplication of cultures and materials viz., plant materials, chemicals and glassware used in this study is presented in this chapter.

Experimental Material

The axillary bud explants used for initiation experiments were collected from mature Pomegranate plant , grown at shed house of Tissue Culture laboratory, Cadila Pharmaceuticals Limited Hirapur Ahmedabad.

Culture Media

Any liquid or solid preperation made specifically for the aseptic growth, storage or transport of plant**.**

Chemicals

The chemicals used in the research work were of research grade and were obtained from Qualigens, ANUCIN and Excel Crop Care.

Culture Bottles

Glass bottles of 250ml were used as culture vessel in experiments. Bottles were properly cleaned and oven dried before use.

Preparation of Media

In the present investigation Murashige and Skoog, 1962 (MS) Media were used as basal salt medium during different studies. Detailed composition of these media is given in the table below:

Compostion of basal medium (Murashige and Skoog, 1962.)

CONSTITUENT	CONCENTRATION(mg/lit)
MACRO ELEMENTS	
Ammonium Nitrate	1650
Potassium Nitrate	1900
Calcium Chloride. 2H2O	440
Magnesium Sulphate.7H2O	370
PotassiumDihydrogen Phosphate	170
MICRO ELEMENTS	
Boric acid	6.2
Potassium Iodide	0.83
Sodium Molybdate	0.25
Cobalt Chloride. 6H2O	0.025
Manganese Sulphate.4H2O	22.3

Zinc sulphate.7H2O	**8.6**
Copper Sulphate. 5H2O	**0.025**
Ferric sulphate	**27.8**
Na2EDTA	**37.3**
VITAMINS	
Nicotinic Acid	**0.5**
Pyridoxine hydrochloric acid	**0.5**
Thiamine hydrochloric acid	**0.1**
Glysine	**2**
Myo Inositol	**100**

Different growth hormones were added to MS basal media, alone or in combinations to check their effect on growth and development of Pomegranate under in vitro.

Changed Growth Hormones used in Shooting and Rooting

CONSTITUENTS AND CONCENTRATION (mg/lit)

TRIAL CODE	IAA	IBA	BAP	NAA	AgNO3	ADS
B1	0.1	0.125	------	-----	-----	-----
B2	0.2	0.125	------	------	------	------
B3	0.3	0.125	------	------	-----	------
B4	0.1	0	-----	----	------	-----
B5	0.2	0	------	-------	-----	------
B6	0.3	0	------	------	-----	------
B7	0.125	-----	------	0.1	------	-----
B8	0.125	------	------	0.2	------	------
B9	0.125	------	------	0.3	------	----
MS1	------	------	-------	-----	1	------
MS2	-----	-----	1.8	0.9	1	30
MS3	------	-------	0.4	0.3	------	------

Growth hormones added for Multiplication and Rooting 3 Trials are done each containing 30 bottles were prepared according to the composition given above and were stored at 7-8 degree centrigate. The pH of media was adjusted to 5.8-0.1 using 0.1N sodium hydroxide or 0.1N hydrochloric acid.

MICROPROPAGATION

Micropropagation is a method of propagating plants that uses very small plants grown in sterile area. This process is a continuous process but it is divided into different stages. Murashige proposed four different stages that can be adopted for overall production technology of clone commercially. Stages 1-3 are followed under in vitro condition, whereas stage 4 is accomplished in a green house environment. **Deberg**

and Maene suggested an additional stage 0 for various micro propagating systems. The adoption of all these stages not only simplifies the daily operation, accounting and product cost , but also allows for greater ease in communication with other laboratories. Micropropogation procedure is divided in stages for the sake of understanding. Murashige proposed three stages (1 to 3) stages, **Debergh and Maene** added stage 0. Currently we adopted 4 stages procedure (0-4).

METHODS

Stage 0: Selection and maintenance of stock plant for culture initiation

This stage was basically introduced to overcome the problem of contamination. Stock plants are grown under more hygienic conditions to reduce the risk of contamination.

Stage 1 : Initiation and establishment of aseptic culture

Preperation of media:

Media was prepared as per the procedure given

Sterilization of Media and Culture Vessels:

Fixed volumes of boiled medium, after thoroughly dissolving agar, poured in glass bottles. After closing with polypropylene caps, the vessels containing media i.e. glass bottles were sterilized by autoclaving at a pressure of 15 psi and 121 degree centrigate for 15

minutes. Steel plates and other tools like scalpels, brown paper, swabbing cloth, forceps, and apron were sterilized at same pressure for 20 minutes.

Aseptic Conditions

All the in vitro operations viz.. surface sterilization of explant, innoculation of explant, subculturing etc.. were carried out on sterile laminar airflow hood. The inner area of laminar air flow was exposed UV light for 30 minutes. Surface of culture vessel was wipping with IPA before expose in laminar air flow. The devices used for innoculation and subculturing (viz.. scalpels, forceps, surgical blade etc..) were frequently heat sterilized using a glass bead sterilizer during different operations under the laminar air flow hood.

Surface sterilization of Explants

Surface sterilization of explant is an important task in plant tissue culture to initiate asepetic cultures, to be used further for in vitro studies. Axillary bud explants of Pomegranate were thoroughly washed with tap water containing few drops of Tween - 20, followed by running tap water treatment for 30 minute. Explants were further treated with antibiotic (streptocycline) at 100 mg/ lit in orbital shaker at 120 rpm for 45 minutes, followed by fungicide(Ridomil) at 1000mg/lit for 20 minutes and transfer to laminar air flow. The axillary bud was surface sterilized with the treatment of 0.1%

HgCl2 for 5 minutes followed by four washes with RO water.

Innoculation and Incubation of Explant.

Surface sterilized explants were inoculated (3 explants/ 1 bottle) into 250 ml sterilized culture bottle containing Murashige and Skoog (1962) medium with 0.6% agar and then incubated in culture room for in vitro shoot initiation at 26+- 2°C temperature, 50-60% relative humidity and 16 hours photoperiod of 65.33 micro mol m-2 s-1 fluorescent cool light were used for different experiments under study.

Stage 2 :

Induction of Shoot.

Effect of various phytohormones on shooting was studied using explants viz.. axillary buds aseptically grown on 3 different media. Various Auxin and Cytokinin (IAA,IBA,BAP,NAA,SilverNitrate,AdenineSulphate) in combinations or alone were incorporated in Murashige and Skoog (1962) medium along with 3% sucrose and 0.8% agar. Medium containing Mrashige and Skoog (1962) along with phytohormones Calcium Nitrate, Adenine hemisulphate, BAP, Kinetin with 3% sucrose and 0.8% agar was kept as a check control (Medium Code - MS0).

Medium MS1 contains Silver Nitrate(1 mg/lit) in Murashige and Skoog (1962) basal.
MS2 contains BAP, NAA, Silver Nitrate, Adenine Sulphate in Murashige and Skoog Media (1962).
MS3 contains NAA, BAP in Murashige and Skoog Media (1962).

Stage 3 : Rooting of regenerated shoots or germination of somatic embryos in vitro

In this stage, shoots or shoot clusters from stage 3 are prepared to transfer to rooting media. Shoots are seperated manually from clusters and transferred on a rooting medium containing IAA,IBA,and NAA. Elongation of shoots prior to rooting, rooting of shoots, and prehardening cultures to improve survival are some of the activites carried under this stage.

Stage 4: **Hardening**

Transfer of plantlet to sterilized soil for hardening under green house environment.

Crieteria to be Observed are:

Shoot Quality
Leaves Quality
Leaves Proliferation
Average number of leaves
Percentage of Shoot Regeneration
Length of Shoot

Percentage of root induction
Number of roots
Length of root

Advantages of Micropropagation

1. The chief advantges of micropropagation is the extremely high multiplication rates, e.g 106 plants/ year from a single explant.
2. Very small explant can be used for micropropagation, which is impossible with conventional technique.
3. During micropragation , fungi, bacteria are usually eliminated so that the plants obtained are clean, while conventional methods propagate the disease as well.
4. Plants can be maintained in vitro in a pathogen free environment
5. Micropropogation can be carried out throughout the year independent of seasons

Disadvantages of Micropropagation

1. It is very expensive and can have a labour cost of more than 70%
2. As monoculture is produced after micropropagation, in case of infection whole crops can get damaged.
3. An infected plant sample can be produced after infected progeny
4. Not all plants can be successfully tissue cultured, often because the proper medium for growth is not known or the plants produced secondary metabolic chemicals that stuny or kill the explant.

5. Some plants are very difficult to disinfest of fungal organisms.

HARDENING

Green House
Shade House

Green House

A green house is a structure with a glass or plastic roof and frequently glass or plastic walls. It heats up because incoming solar radiation from the sun warm the plants, soils and other things inside the building faster than heat can escape the structure. Air warmed by the from hot interior surfaces is retained in the building by the roof and wall. These structures range in size from small sheds to very large buildings.

Green house can be divided into glass greenhouses and plastic greenhouses. Plastics mostly used are PEfilm and multiwall sheet in PC or PMMA. Commercial glass greenhouses are often high tech production facilites for vegetables or flowers. The glass greenhouses are filled with equipment like screening installations, heating, cooling, lighting and may be automatically controlled by a computer.

The glass used for a greenhouse works as a barrier to air flow and its effect is to trap energy within greenhouse, which heats both plants and the ground inside it. This warm the air near the ground, and this air is prevented from rising and flowing away. This can be

demonstrated by opening a small window near the roof of the green house the temp drops considerably. The principle is the basis of the autovent automatic cooling system. A miniature greenhouse is known as a cold frame.

SHADE HOUSE

A shade house serves the opposite purpose of a green house, it is used to protect cultivated plants from excessive heat, light or dryness.

2 types of Hardening:
Primary Hardening
Secondary Hardening

PRIMARY HARDENING

CONDITION : Temperature - 30-32°C
Humidity - 70-75%
Light- 3000-6000 Lux light with appropriate duration
Sterile condition- No

PROCEDURE :

- The plant after the rooting stage are brought to the green house for hardening.
- The bottles are kept in the green house for about 1-2 weeks prior to actual hardening process starts. From here the process of acclimatization starts. The bottles are kept in the green house so that they can

withstand the condition in the green house which are harsher than lab conditions when the plants are taken out of the bottle.

- After this the plants taken out of the bottle and the agar stuck to the roots is removed manually.
- Then they are washed in 80 TDS R.O. water
- After washing the plants are treated with Carbendazim, which is a synthetic fungicide. This prevents the plant from fungal infection.
- Now the plants are ready for planting.

SORTING

- The plants after Carbendazim treatment are sorted according to size as small, medium and large.
- This is done to keep the plant growth uniform after planting.

PLANTING

COCO peat

- Coco peat is the decomposed form of the coconut coir. The coir is decomposed for 1 year. This decomposed form of coir is called coco peat.
- Coco peat is used for primary hardening as it gives excellent support to the plant as well as it has a very good water holding capacity.
- Coco peat is treated with contact fungicide DM 45 to avoid fungal contamination of the roots.
- Coco peat is filled thoroughly in a 96 well pour plate.

The sorted plants are planted into the pour plate filled with coco peat.
(An alternate method of planting is by planting in JIFFY which is small amount of coco peat packed in a muslin cloth)

- Some plants with slow root growth are treated with growth regulators such as Indole 3- Butyric Acid (IBA) and Humic Acid.
- After planting the plant is kept for around 4 to 6 weeks in the green house.
- After this peroid plants are preceded for secondary hardening process.

4 to 6 weeks peroid

During this period the plants are treated periodically with different fungicides and fertilizers for its growth.
Rooting of the plants starts within a week

Shade House

- In shade house the pour plates are arranged in the bed and are covered with plastic to form a tunnel.
- This gives humidity similar to that in green house.
- Moreover the temperature in the tunnel is lowered by pouring water on the plastic sheets of the tunnel with the help of micro sprinklers.

CONDITIONS

Temperature - 35-40°C
Humidity - 60-70%
Light - 25% of sunlight
Sterile condition - No

PROCEDURE

The plants after growing to certain height are planted in the soil.
Composition of Soil
Clay - 50%
Sand-25%
FYM - 25%
After planting the plants are allowed to grow for 4-6 weeks.
After the plants are grown upto the height of 1 foot it is sold to the customers.

4 to 6 weeks period
As in the case of primary hardening, similarly the plants are treated with different fungicides and fertilizers to the plant of their better growth.
Different fungicides which are used are

COC
DM45

CHAPTER 4
OBSERVATION

All observations were recorded from 15 explants in each treatment.

1) Percentage of explants sprouted

The phenomenon of activation and growth of dormant bud which will develop in shoot upto 0.2mm is termed as explant sprouting.
After 28 days of explants innoculation, development of first visible sprout was considered as sprouting. The ratio of number of sprouted explants to the total number of explants innoculated, expressed in percentage, considered as percentage of sprouting.

2) Percentage of shoot induction

Shoot induction became evident when small fresh leaves began to emerge.

Development of first visible leaf considered as shoot induction. The ratio of the number of explants showing shoot induction to the total number of explants innoculated, expressing in percentage, considered as percentage of shoot induction.

3) Average number of leaves

Number of leaves per sprout was counted after 28 days of innoculation for each explant and average out over replication.

4) Average Shoot Length(cms)

Each shoot was measured in centimeters for its length after 2-3 cycle of transfer in multiplication stage. Shoot length increases when wooden parts gets detached from expalnt.

5) Percentage of root induction

After 5-6 cycles of multiplication the explant is transfered to rooting media then after 28 days of innoculation, root appearance on explants considered as root induction. The roots of the number of rooted explants to the total number of explants innoculated, expressed as percentage, considered as percentage of root induction.

6) Average number of roots

Number of roots on each shoots were counted and average out of each treatment

7) Average Root Length

The root length was measured in centimeters for its length after 28 days of innoculation in rooting media for each rooted shoot and average out for average root length.

8) Statistical Analysis

Data on different characters studied were analyzed on mean values. The different statistical procedures followed for analysis were as under.

Arithmetic mean

The arithmetic mean is commonly called the average: When the word mean is used without modifier, it can be assumed that it refers to the arithmetic mean. **The mean is the sum of all the observations divided by the total number of observations.**

$$M=\sum X/N$$

Where,

M = Arithmetic Mean
∑X= sum of observation
N= Total number of observation.

STANDARD DEVIATION

Standard deviation is defined the square root of the variance.

$$s = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

Where,

S= Standard deviation

∑x = sum of observation

∑x2= Square root of all observetion

n = Total no of observation

Standard error

The standard error of the mean is the standard deviation of those sample means over all possible means over all possible sample dawn from the population.

$$SE_{\bar{x}} = \frac{s}{\sqrt{n}}$$

Where,

$\bar{X}$ **= The sample mean**

S.Em= standard error of mean

s = Standard deviation

n = number of observation

CHAPTER 5
RESULTS AND DISCUSSION

In the present investigation, strength were made to study the effect of phytohormones on various aspects of shoot and root growth. The development of efficient and reproducible regeneration protocol from tissue is prerequisite for the successful application of micropropogation technology for the mass multiplication of plant. Various concentration and combinations of auxin and cytokinin were used in MS(1962) medium and observed for the morphogenic responses of explants of Pomegranate.

The results of each of these aspects are presented and discussed here with considering weekly observations and pooled observations for different characters under study.

Percentage of explants sprouted

The phenomena of activation and growth of dormant bud, which will develop in shoot is termed as explants sprouting.

Week wise Percentage of explants sprouted

	CONCENTRATION (mg/lit)		Percentage of explant sprouted			
TREATMENT	AgNO3	ADS	WEEK1	WEEK 2	WEEK3	WEEK4
T1	0.00	0.00	30.33	0.00	10.00	0.00
T2	0.5	10	38.88	10.00	40.00	0.00
T3	1.0	20	40.00	33.33	20.00	2.22
T4	1.5	30	33.00	0.00	33.00	0.00

During 1st week of explant innoculation, Treatment T2 (38.88%) and Treatment T3 (40%) showed maximum number percentage of explant sprouting followed by T4 (30%), Whereas, lowest percentage of explant sprouting was observed on treatment T1(30.33%).

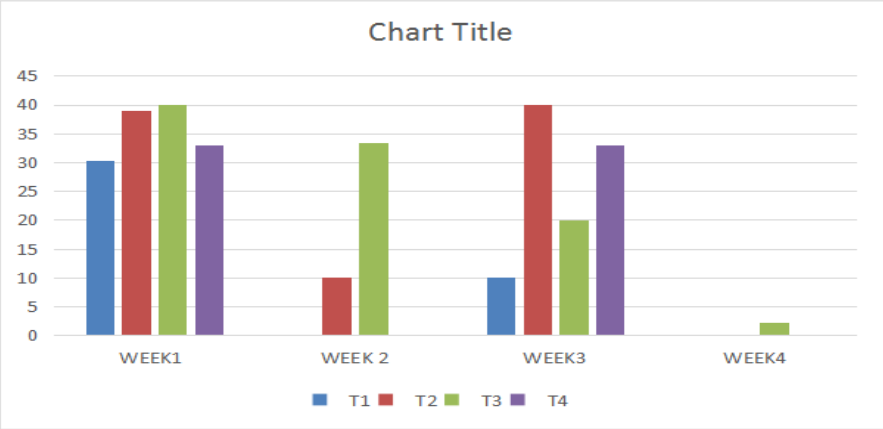
In 2nd week, highest percentage of explants sprouting was obtained in T3 (33.33%). In Treatment T2, (10%) explants were sprouted, while Treatment T1 and T2 showed no percentage of explant sprouting.

The data on percentage of explant sprouting collected in 3rd week revealed that the highest percentage of explant sprouting was found in treatment T2(40%). In Treatment T4 (33%) explant sprouting was obsereved.In treatmet T1 (10%) and T3 (20%) were recorded.

Overall weeks highest percentage of explant sprouting was observed in Treatment T3 (95.55%) followed by Treatment T2 (88.88%) and Treatment T4 (66%). Lower percentage of explant sprouting was obtained in Treatment T1 (40%).

Data pertaining to percentage of explants sprouting revealed that percentage of in the medium influence the trait explants sprouting. **Islam et al. (1994)** reported that presence of cytokinin promoted the shoot induction alone or in combination with auxin , while presence of auxin alone was adversely affected on sprouting of explants. Presence of only auxin in medium gave lowest sprouting indicated, cytokinin has positive effect on differentiation of axillary bud as compared to auxin.

SPROUTING



X axis : Treatment
Y axis: % of sprouting

Graph showing percentage of sprouting over four weeks

Percentage of shoot induction

Shoot induction become evident when small fresh leaves began to emerge.

Week wise Percentage of shoot induction

Treatment	Concentration				Percentage of shoot induction			
	BAP	NAA	AgNO3	ADS	WEEK1	WEEK2	WEEK3	WEEK4
T1	0	0	0	0	0.00	2.22	10.00	21.42
T2	1.0	0.6	0.5	10	0.00	6.57	18.00	20.07
T3	1.8	0.9	1	20	0.00	12.75	37.66	48.00
T4	2.0	1.0	1.5	30	0.00	10.55	15.75	12.33

During first week of cultures initiation, no shoot induction obtained.

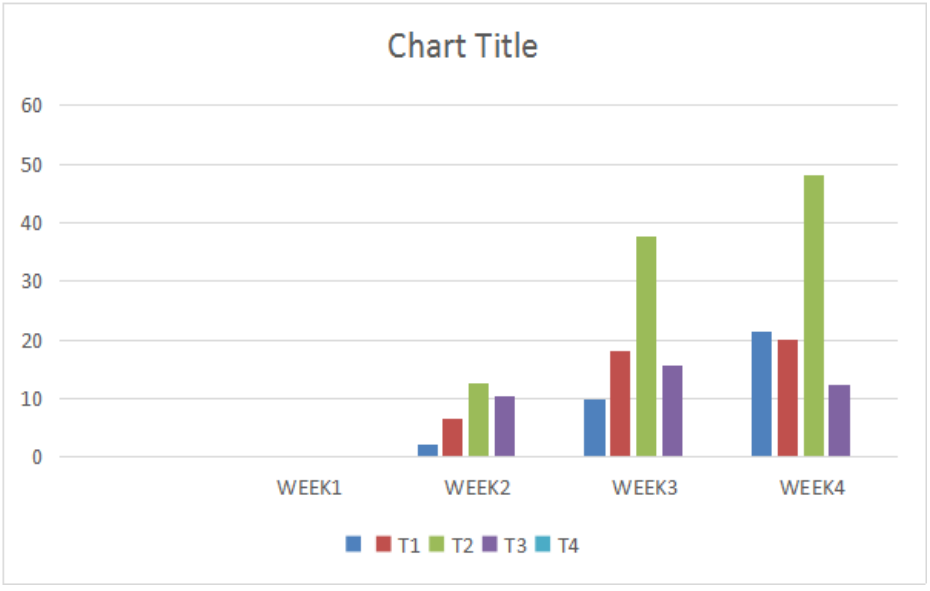
The data on percentage of shoot induction of shoot induction collected in 2nd week revealed that the highest percentage of shoot induction was observed in T3 (12.75%). The lowest percentage of shoot induction was found in treatment T1(2.22%).In both other treatments, T2 and T4 , (6.57 and 10.55%) is observed.

In 3rd week, highest percentage of shoot induction was observed in T3(37.66%). In treatment T2 (18%) and Treatment T4 is (15.75%) while treatment T4 showed lower percentage of explant sprouting (10%).

In 4th week highest percentage of shoot induction was found in T3(48%). In Treatment T1(21%) and T2 (20%) .Lowest percentage of shoot induction was obtained in Treatment T4 (12.33%).

In this study cytokinin alone induced shoots as reported by Yoshika et.al (2006). Auxin does not give high number of shoot induction alone or in combination of cytokinin may be due to its negative role in auxillary sprouting.

Shoot Induction



Graph showing percentage of shoot induction over four weeks

Average number of leaves

Week wise Average number of leaves

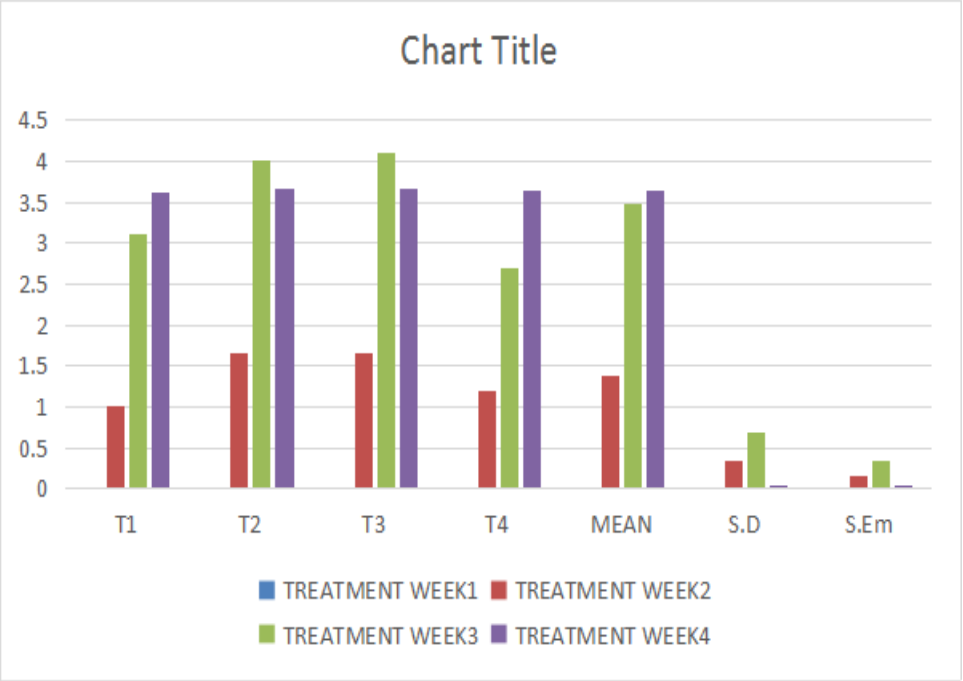
TREATMENT	CONCENTRATION		Average number of leaves			
	BAP	NAA	WEEK1	WEEK2	WEEK3	WEEK4
T1	0.00	0.00	0.00	1.00	3.10	3.63
T2	-	0.2	0.00	1.66	4.00	3.66
T3	0.3	-	0.00	1.66	4.11	3.66
T4	-	0.4	0.00	1.20	2.69	3.64
MEAN			0.00	1.38	3.48	3.65
S.D			0.00	0.33	0.69	0.01
S.Em			0.0000	0.1667	0.3458	0.0075

During first week of cultures there is no leaf growth was observed. During 2nd week of explant innoculation, both the treatments, T2 and T3 showed maximum number of leaves (1.66) followed by T4 (1.20), whereas, lowest number of leaves was counted on Treatment T1(1.00).

The data of the above table revealed that in 3rd week highest number of leaves was observed in Treatmnet T3 (4.11). In Treatmnet T1 (3.10) in Treatmnet T2 (4.00) leaves were found, while Treatment T4 showed lowest number of leaves (2.69).

In 4th week, the highest number of leaves were counted in Treatment T3 and T2 (3.66) followed by T4 (3.64).Lower number of leaves was obtained in Treatmnet T1(3.63).

There was very narrow difference between the effects of all the combinations on number of leaves. However on the basis of available difference, cytokinin and auxin alone induces leaves proliferation Role of auxin in leaf development is reported by Arney, S.E.(1988).



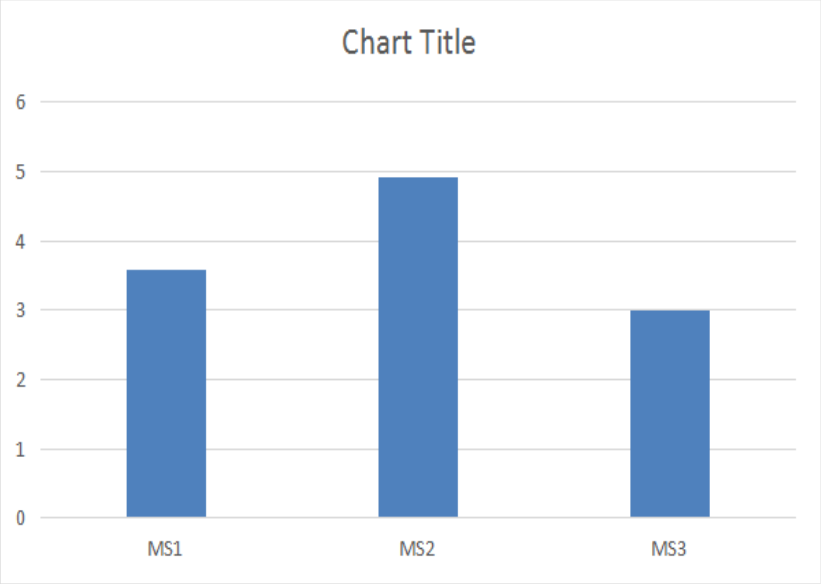
Yaxis: Average no of leaves
Xaxis: Treatment

Graph showing average number of leaves over four weeks

Average shoot length

The data on average length of shoot were collected over all week revealed that the highest length of shoot was observed in Treatment T3(4.92cm) followed by Treatment T2 (3.57cm) and Treatment T2(3cm). The lowest length of shoot was obtained in Treatment T1 (1.38cm).

Addition of cytokinin gave longer shoot than in combination with auxin or auxin alone **(Nabi S.A. et.al 2002).**



Y axis: Average shoot Length
X axis: Treatment

Graph showing average shoot length over four weeks

SHOOT LENGTH

Percentage of root induction

Week wise Percentage of root induction

Treatment	Concentration		Percentage of root induction			
	IAA	IBA	WEEK1	WEEK2	WEEK3	WEEK4
T1	0.1	0.125	0.00	10.5	20.8	60.3
	IAA	NAA				
T2	0.1	0.125	0.00	0.00	25.7	63.6
T3	0.2	0.125	0.00	8.09	22.2	52.2
T4	0.3	0.125	0.00	10.00	33.3	47.0

During first week no root induction was obtained.
The data on percentage of root induction recorded from 2nd week revealed that the highest percentage of root induction was observed in T1(10.05%). In Treatment T4 percentage of root induction was (10%), followed by Treatment T3 (8.09%). No root induction was found in Treatment T2.

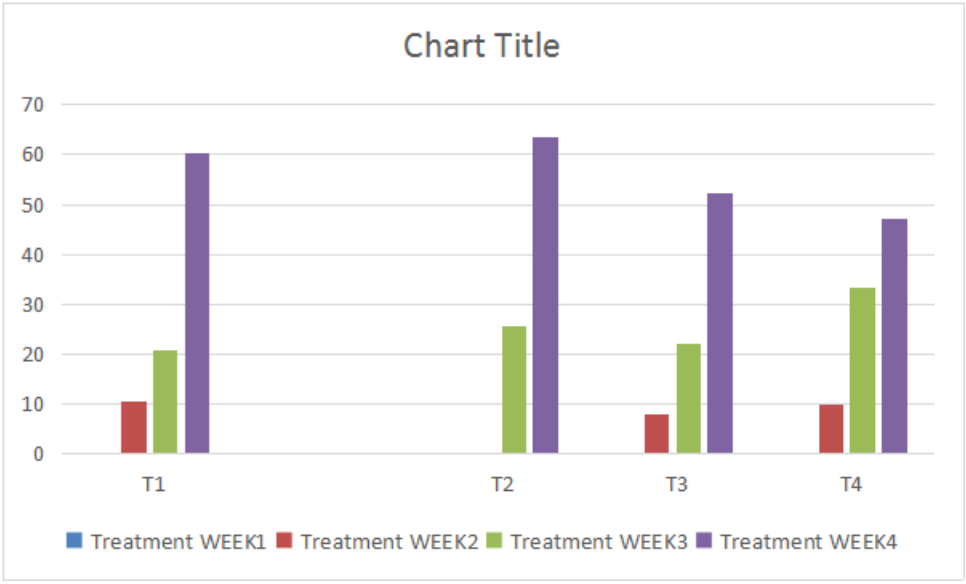
The data on percentage of root induction recorded in 3rd week revealed that highest percentage of root induction was observed in treatment T4 (33.3%). In treatment T2 (25.7%) roots were found , while Treatment T1 and T3 showed lowest number of roots (20.8% and 22.2%).

After 4 weeks of incubation, the highest percentage of root induction was observed in Treatment T2(63.6%) followed by T1 (60.3%) and (52.6%).

Treatment T4 showed (47.0%) gave the lowest count for percentage of root induction.

In this experiment, clear effect of auxin on root induction was observed, which was also reported by Taiz, L. and Zeiger, E (1998).

Cytokinin supressed rooting as repoorted by Anon.(2006) and gave lowest count for root induction alone or in combination with auxin in prresent study.



Y axis: % of Root Induction
X axis: Treatment

Graph showing Percentage of Root induction over 4 treatment

Root Induction without callus

Average number of root

Week wise average number of roots

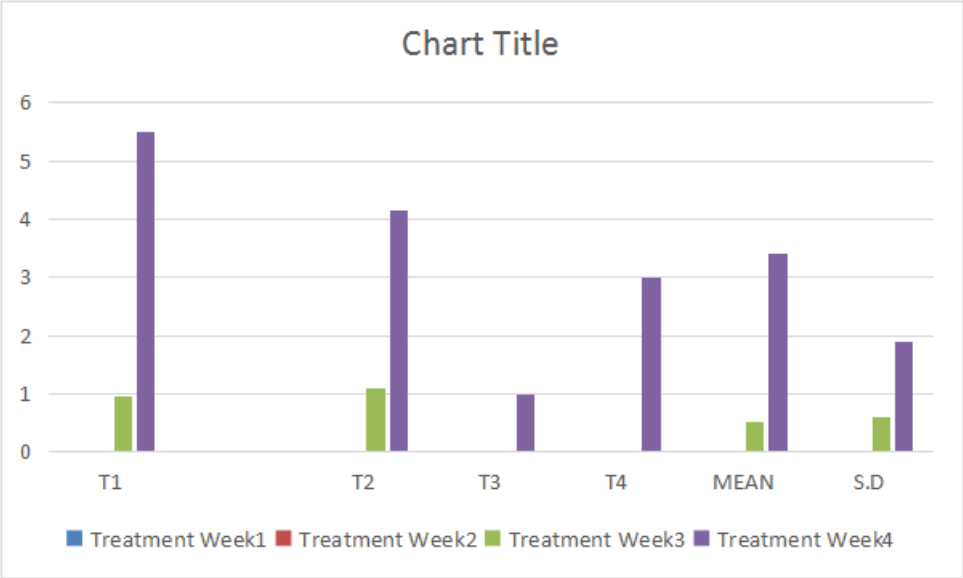
Treatment	Concentration(mg/lit)		Average no of roots			
	IAA	IBA	Week1	Week2	Week3	Week4
T1	0.1	0.125	0.00	0.00	0.95	5.50
	IAA	NAA				
T2	0.1	0.125	0.00	0.00	1.11	4.16
T3	0.2	0.125	0.00	0.00	0.00	1.00
T4	0.3	0.125	0.00	0.00	0.00	3.00
MEAN			0.00	0.00	0.51	3.41
S.D			0.00	0.00	0.59	1.90

During first and second week of cultures no roots was obtained.

The data on average no of roots showed in 3rd week, the highest number of root was observed in Treatment T2(1.11) followed by Treatment T1 (0.95). Treatment T3 and T4 showed no root development after 3rd week of culture growth.

Overall weeks, the highest average number of roots was observed in Treatment T1 (5.50) followed by T2 (4.16) and T4 (3.00) and the lowest number of roots were observed in Treatment T3 (1.00).

In present study, auxin promoted root growth, which is also reported by Anon (2006).



Y axis: Average no of roots
X axis: Treatment

Graph showing average no of roots over four treatment

Average Root Length.

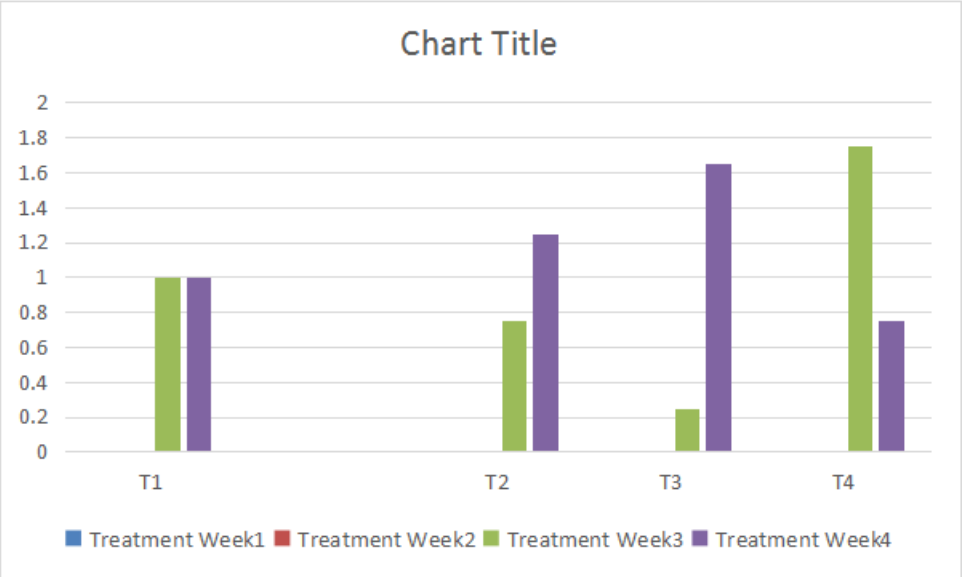
Week wise Average root length

Treatment	Concentrations (mg/lit)		Average Root Length			
	IAA	IBA	Week1	Week2	Week3	Week4
T1	0.1	0.125	0.00	0.00	1	1
	IAA	NAA				
T2	0.1	0.125	0.00	0.00	0.75	1.25
T3	0.2	0.125	0.00	0.00	0.25	1.65
T4	0.3	0.125	0.00	0.00	1.75	0.75

During 3rd week the shortest root were induced by Treatment T3 ie (0.25cm) followed by treatment T2 (0.75cm). Treatment T1 and T4 showed the highest i.e. 1cm and 1.75cm.

Data from the above table for average root length, pooled over the week showed that the highest length of root was observed in Treatment T3 (1.65cm), followed by T2 (1.25cm) and T1(1cm). Smallest root length is observed in Treatment T4 (0.75 cm).

Both auxin and cytokinin have synergetic effects on development of roots. Auxin played the role in induction of roots whereas cytokinin promotes growth of root.



Y axis: Average root length
X axis: Treatment

Graph showing average root length over four weeks

ROOT LENGTH

CHAPTER 6
SUMMARY AND CONCLUSION

The present exploriation entitled " IN VITRO PROPOGATION OF POMEGRANATE
from axillary bud was carried out at Tissue Culture Laboratory, Cadila Pharmaceuticals limited, Hirapur, and Ahmedabad during January- May 2018. The experiment was conducted with a view to study in vitro establishment of axillary bud with responses of phytohormones.
The characters studied were Percentage of Sprouting, Percentage of Shoot Induction, Average number of leaves, Length of shoot, Percentage of root induction, Number of roots , Length of root etc..

In present experiment axillary bud of pomegranate was used as explant for different experiments. Based on the purpose of study, Murashige and Skoog (1962) medium were used as basal media for different studies.

Different Phyohormones I.e. IAA, IBA, BAP, NAA, AgNO3, ADS were added in basal media . Salient findings of the study were summarized as follows.

Effect of growth hormones on axillary bud

Effect of various phytohormones on axillary bud was studied. Different growth hormones I.e. Auxin (IAA) and cytokinin (IBA)
Were incorporated in Murashige and Skoog (1962) basal media along with 3% sucrose and 0.8% agar.While medium containing basal salts with 3% sucrose and 0.8%

agar was kept as a control. Different combination and concentration of phytohormones

Percentage of explant sprouted

The highest percentage of explant sprouting was observed in Treatment T3 (95.55%) followed by Treatment T2 (88.88%) and Treatment T4 (66%). Lower percentage of explant sprouting was obtained in Treatment T1 (40%).It indicates that hormones are required in sprouting of explants.

Percentage of shoot induction

The effectiveness of BA was proved to be superior to auxin with respect to shoot proliferation.Medium T3(MS+ 1.8BAP+ 0.9NAA+1AgNO3+20ADS) was the best media for early induction of shoot. The lower percentage of shoot induction was obtained in Treatment T1(33%).

Average number of leaves

The highest number of leaves was counted in treatment T3 and T2 (3.66). Lower number of leaves were obtained in Treatment T1 (3.63). Though, there was not considerable difference between counts BA and IAA alone were better than in combination.

Average shoot length

Medium T3 (MS+ BAP 1.8 mg/lit+NAA 0.9 mg/lit+AgNO3 1 mg/lit+Adenine Sulphate 30 mg/lit) was the best media for highest shoot length. The lowest shoot length was obtained in Treatment T1(1.38cm)

Percentage of root induction

The effectiveness of IAA was proved superior to cytokinin with respect to root induction. Medium T1 (MS+0.1 IAA+0.125 IBA) was the best media for early induction of roots. The lower percentage of root induction was obtained in Treatment T4 (80.3%).

Average number of root

The highest number of roots were induced in Treatment T1 I.e 5.5 whereas the lowest average number of roots was observed in T3 I.e (1.0). Addition of auxin helps in induction of roots, whereas cytokinin has negative effect on root growth.

Average root length

Data for average length, pooled over weeks showed that the highest length of root was observed in Treatment T4 (2.5cm) while the shortest length of root was observed in Treatment T3(1.9).

CONCLUSION

- Significant differences among the phytohormones for various characters studied under in vitro conditions revealed that the expressions of the characters was affected by phytohormones.
- Among the different growth hormones, cytokinin was found to be superior over auxin for shoot induction, $AgNO_3$ is responsible for clumps formation.

- Moreover, cytokinin took the least days of explant sprouting.
- Higher level of auxin induced early root induction, number of root and percentage of axillary root.

CHAPTER 7
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