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IN VITRO STUDY ON FLORAL PARTS OF POMEGRANATE *PUNICA GRANATUM* L. CV. 'GANESH'

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ABSTRACT

In vitro studies were undertaken with a view to study the regeneration potentiality of different floral explants of field grown mature plants. The primary and secondary cultures of segments of petals, entire filaments and anthers induced embryogenic callus on Murashige and Skoog's media supplemented with individual and combined plant growth regulators such as NAA and TDZ. The callus obtained on media containing individual NAA was more in mass and friable. Such callus was used for callus maintenance and initiation of suspension cultures. Subculture studies of callus on semisolid nutrient media supplemented with natural additives like casein hydrolysate (25mg/l, 50mg/l,100mg/l) coconut water (10%,15% and 20%) and Abscisic acid (1mg/l,2mg/l, 3mg/l), revealed the presence of secondary embryogenesis on media. On the contrary, from the embryogenic callus induced on media containing combined PGRs, developed precociously germinated somatic embryos on media supplemented with activated charcoal (2%) and adenine sulphate (4mg/l) and coconut water (15%). The potentiality of embryogenic callus induction, precocious germination of somatic embryos, varied among the different explants depending upon the added growth regulator.

KEYWORDS: NAA and TDZ.

INTRODUCTION

In vitro technique has allowed investigators to study the regeneration potentiality of both juvenile and mature plant parts of Pomegranate by offering knowledge for mass multiplication of elite cultivars, selection of somaclonal variants, synthetic seed production, and new cell lines for genetic transformation and fruit crop improvement. The Pomegranate,

Punicagranatum L., an ancient, mystical and highly distinctive fruit, is the predominant member of two species comprising the Punicaceae family. It was lauded in ancient times in the Old Testament of the Bible, the Jewish Torah, and the Babylonian Talmud as a sacred fruit conferring powers of fertility, abundance, and good luck. It features prominently in the ceremonies, art and mythology of the Egyptians and Greeks and was the personal emblem of the Holy Roman Emperor, Maximilian. Pomegranate is the symbol and heraldic device of the ancient city of Granada in Spain, from which the city gets its name. The genus name, Punica, was the Roman name for Carthage, where the best pomegranates were known to grow. Pomegranate is known by the French as grenade, the Spanish as granada, and literally translates to seeded ("granatus") apple ("pomum"). The Pomegranate tree typically grows 12-16 feet, and can be extremely long lived, as evidenced by trees at Versailles, France, known to be over 200 years old. The leaves are glossy, lance shaped and the bark of the tree turns gray as the tree ages. The flowers are large, red, white, or variegated and have a tubular calyx that eventually becomes the fruit.

The present study has been carried out on Pomegranate with following objectives: To

1. Establish organ cultures from floral parts.
2. Study the individual and combined effect of auxin- NAA, Picloram, 2,4-D and cytokinin-TDZ, on regeneration potentiality of explants collected from field grown mature plants.
3. Study the maturation and germination of somatic embryos from embryogenic callus.
4. Study anti-oxidant activity of aqueous extract and evaluate antibacterial, antifungal activities of petals, petal induced fresh callus and dried callus.

Pomegranate: The fruit of paradise

The Pomegranate, botanical name Punicagranatum, is a fruit-bearing deciduous shrub or small tree growing between 5–8 meters (16–26 ft) tall. The pomegranate is widely considered to have originated in Iran and has been cultivated since ancient times.

MORPHOLOGY

Vegetative growth

Pomegranate is a shrub that naturally tends to develop multiple trunks and has a bushy appearance. When domesticated, it is grown as a small tree that grows up to 5m. Under natural conditions, it can sometimes grow up to more than 7m; at the other extreme, in severe natural environment, one can find creeping bush varieties. In addition, there are dwarf cultivars that do not exceed 1.5m.

Most of the Pomegranate varieties are deciduous trees. However, there are several evergreen Pomegranates in India. Singh et al. (2006) reported deciduous Indian varieties and identified 16 genotypes that behaved as evergreen in Rajasthan India. Sharma and Dhilom (2002) evaluated 30 evergreen cultivars in Punjab India. There are clearly prominent differences among Pomegranate varieties with respect to leaf shed. Some evergreen cultivars shed their leaves in higher elevations and colder climates and should be regarded as conditionally deciduous.

The young branches from the vegetative growth of the recent year are numerous and thin. The color of the bark of young branches depends on the variety. In some, bark color varies from pink to purple, while in others it is light green with pink-purple spots or stripes. Upon maturation, the pink color of the branch starts to disappear, and in the second year, the bark will become light gray that darkens as the tree mature. The bark of the old tree tends to split, and in certain cases it is detached from the trunk. The wood color is light yellow. Young branches sometimes have thorns at their tips that are visible already in the axils in the young bloom. The young branches are polygonal (quadrangular). As the branches mature, they become round. Young leaves tend to have a reddish color that turns green when the leaf matures. In varieties with young pink-purple bark, this color appears also on the sheath and the petiole, on the lower part of the central vein, and in the leaf margins.

Leaf Morphology

Leaves have an oblanceolate shape with an obtuse apex and an acuminate base. Mature leaves are green, entire, smooth, and hairless with short petioles. They usually have a special glossy appearance (particularly at the upper part of the leaf) and contain idioblasts with secretory substances that have not yet been identified. The leaves are exstipulate, opposed and pairs alternately crossing at right angles. Some varieties have 3 leaves per node arranged at 120 degrees and even 4 leaves per node on the same tree (2 opposed leaves per node).

Flower

Flowering occurs about 1 month after bud break on newly developed branches of the same year, mostly on spurs or short branches. Flowers can appear solitary, pairs, or clusters. In most cases, the solitary flowers will appear on spurs along the branches while the clusters are terminal. In the northern hemisphere, flowering occurs in April-May. However, flowering may continue until end of summer, particularly in young trees. Such flowers are fertile, but the fruit will not properly mature because the trees enter the cooler season and the dormancy

period in Mediterranean climatic conditions. Flowering and the consequent fruit set last about 1 month. During this period, there are three waves of. In evergreen cultivars in southern India, flowering season was observed in three periods: June, October, and March or throughout the year.

Fruit

The fruit develops from the ovary and is a fleshy berry. The nearly round fruit is crowned by the prominent calyx. The apex of this crown is almost closed to widely opened, depending on the variety and on the stage of ripening. The fruit is connected to the tree with a short stalk. Following fruit set, the color of the sepals' skin in the developing fruit changes continuously from the prominent orange-red to green. In later stages of fruit maturation, the color will change again until it reaches its final characteristic color as the fruit ripens. The external color ranges from yellow, green, or pink overlain with pink to deep red or indigo to fully red, pink or deep purple cover, depending on the variety and stage of ripening There are some exceptional cultivars, such as the black pomegranate which acquires its black skin very early and remains black until ripening time.

MATERIALS AND METHODS

Murashige and Skoog's medium (Murashige and Skoog, 1962) with 2.0 per cent sucrose and 0.8 per cent agar was used as the basal medium (MS media). Only analytical reagent grade (Analar) chemicals and Borosil glassware were used. The cleaning of glassware was done by treating them with 40 per cent chromic acid overnight and rinsing in tap water. After thorough washing with teepol (5 per cent), they were rinsed with distilled water and dried in an oven at 120° C for 2 hours.

For making the nutrient medium, separate stock solutions were prepared (Table 1). The following supplements were incorporated into the medium either individually or in a few combinations before the final volume was made.

1. Plant growth regulators (PGR's)
2. Casein Hydrolysate (50mg/l, 100mg/l)
3. Coconut water (10%, 15%, 20%)
4. Activated charcoal (2%)
5. Abscisic acid
6. Adenine sulphate

7. Ascorbic acid (25mg/l)

The pH of the medium was adjusted to 6.0 with 0.1 NaOH or HCl using digital pH meter. About 15ml of the medium was dispensed to each of the culture tubes (150mm length, 25mm diameter) or 30ml to flasks (100ml). After proper covering, they were autoclaved at 121°C, 15lbs, pressure for 20 min. For each experiment a set of 12 cultures was made and each experiment was repeated twice. All the cultures were maintained under diffused light (1000 lux) for approximately 10 hours daily at 25 ± 2° C and 50-60 per cent relative humidity.

Table no. 1: Murashige and Skoog's basal medium (1962).

Stock Solutions	Constituents	Concentration (in mg)	Volume Of Stock Solution (in ml/l)	Final Concentration Of Medium (in mg/ml)
STOCK A (20X) Inorganic salts, major or macro nutrients	Ammonium nitrate	33000	50ml	3300
	Potassium nitrate	380000		3800
	Calcium chloride	9600		960
	Magnesium sulphate	7400		740
	Potassium dihydrogenortho phosphate	3400		340
STOCK B (200X) Micronutrients	Potassium iodide	166	5ml	0.83
	Boric acid	1,240		6.2
	Manganese sulphate	4,460		22.3
	Zinc sulphate	1,720		8.6
	Sodium molybdate	50		0.25
	Copper sulphate	5		0.025
	Cobalt chloride	5		0.025
STOCK C (200X) Iron supplements	Ferros sulphate	5,570	5ml	27.85
	EDTA	7,450		37.25
STOCK D (200X)	Inositol	20,000	5ml	100
	Nicotinic Acid	100		0.5
	Pyrrodoxin HCl	100		0.5
	Thymine HCl	20		0.1
	Glycine	400		2.0
SUGAR	Sucrose	30gm		
pH	Adjusted by 1N NaOH/HCl	6.0		

Title No. 1: Individual Effect of Plant Growth Hormones on Floral Parts

To study individual effect of PGR's such as NAA, TDZ, Picloram and 2,4-D on petals, filaments and anthers of *Punica granatum.*

Requirements

1. Chemicals: chemicals required for surface sterilization, stock solutions, ascorbic acid (25mg/l)
2. Plant growth regulators: 1. NAA(1.0mg/l, 2.0mg/l, 3.0mg/l)

2. TDZ (1.0mg/l, 2.0mg/l, 3.0mg/l)

3. Picloram (0.5mg/l, 1.0mg/l, 2.0mg/l)

4. 2,4-D (0.5mg/l, 1.0mg/l, 2.0mg/l)

3. Glasswares, instruments.

Plant material

Young buds (1cm) obtained from field grown mother plant.

Procedure

1. Procedure for surface sterilization

Wash the buds (1cm) thoroughly with tap water

↓

2% fungicide – 5min- wash with distilled water

↓

2% cetrimide detergent – 5min – washes with distilled water

↓

Sodium hypochloride – 9min – transfer to laminar air flow hood and wash for 3-4 times with autoclaved distilled water

↓

Absolute alcohol – wash- decant thoroughly on sterile filter paper

2. Procedure for initiation of culture

1. Surface sterilized buds were transferred in the laminar air flow hood.
2. The buds were cut open using sterile scalpel and blade.
3. Petals were separated and inoculated in semi-solid medium having various PGR concentrations.
4. Similarly anther and filament were inoculated together without separation in the semi-solid medium.
5. The culture vessels were incubated in growth room, both in light as well as dark.

Title No. 2: Combined PGR Effect on Floral Parts of *Punica granatum*

To study combined effect of auxin and cytokinin; NAA and TDZ using various concentrations on the petals, anthers and filaments of *Punica granatum.*

Requirements

1. Murashige and Skoog's basal medium supplemented with NAA and TDZ along with Ascorbic acid (25mg/l) as an anti-oxidant.

A B

Table 2: Various concentrations of NAA & TDZ used for studying combined effect of NAA and TDZ.

TDZ / NAA	1mg/l	2mg/l	3mg/l
1mg/l	1mg/l 1mg/l	2mg/l 1mg/l	3mg/l 1mg/l
2mg/l	1mg/l 2mg/l	2mg/l 2mg/l	3mg/l 2mg/l
3mg/l	1mg/l 3mg/l	2mg/l 3mg/l	3mg/l 3mg/l

Procedure

1. Standard surface sterilization of small sized (1cm) buds using chemical sterilents.
2. Aseptic inoculation in semi-solid medium (MS medium) supplemented with different combinations of NAA and TDZ.
3. Incubation of cultures in growth room in both light as well as dark conditions.

Title No. 3: Comparative study of different Natural Extracts on Embryogenic Callus obtained from Individual Growth Regulators

Comparitive study of different natural extracts Abscisic acid (1mg/l,2mg/l,3mg/l), Coconut water (10%,15%,20%) and casein hydrolysate (20 mg/l, 25 mg/l, 30mg/l), on germination of somatic embryos from embryogenic callus obtained from individual effect of NAA and TDZ

Requirements

1. Media

a) MS medium supplemented with NAA (0.5mg/l), TDZ(0.5mg/l)

b) Natural extracts: Abscisic acid (1mg/l, 2mg/l, 3mg/l), Coconut water (10%, 15%, 20%), casein hydrolysate (20mg/l, 25mg/l, 30mg/l)

c) Agar (0.8%)

d) sucrose-30gm

2. Instruments, glassware, equipment for inoculation of embyogenic callus under aseptic conditions.

Procedure

1. Embyogenic callus (50mg) obtained from individual effect of NAA and TDZ were aseptically transferred to semisolid medium containing different combinations of Abscisic acid, Coconut water and Casien hydrolysate.
2. A small amount of embyogenic callus (10mg), was transferred into FAA(Formaline Aceto Alcohol) and further used for cytology studies.
3. Cultures were maintained both in light and dark conditions.

Title No. 4: Effect of Coconut Water, Activated Charcoal and Adenine Sulphate on germination of Embryogenic Callus

To study the individual and combined effect of Coconut water (15%), Activated charcoal (2%) and Adenine Sulphate (4mg/l) on germination of somatic embryos from embyogenic callus obtained from combined effect of NAA and TDZ.

Requirements

1. Media: MS Medium without growth hormone
2. Supplements: a) Coconut Water- (15%)

b) Activated Charcoal (2%)

c) Adenine Sulphate (4mg/l)

3. Glassware, instruments and other equipment

Procedure

1. Embyogenic callus (50mg) obtained from combined effect of NAA and TDZ were aseptically transferred to semisolid medium containing different combinations of coconut water, activated charcoal and adenine sulphate.
2. A small amount of embyogenic callus (1mg) was transferred into FAA and further used for cytology studies.

Title No. 5: Maintenance of Callus on Semi-Solid Medium

To maintain non friable mass of callus on MS semisolid medium supplemented with casein hydrolysate (50mg/l and 100mg/l) and individual PGR's.

Requirements

1. Media: MS medium supplemented with:

a) Casein hydrolysate – 50mg/l, 100mg/l

b) NAA- 1mg/l, 2mg/l, 3mg/l

c) TDZ- 1mg/l, 2mg/l, 3mg/l

2. Ascorbic acid(antioxidant)- 25mg/l
3. Glasswares, instruments and equipment required for aseptic inoculation.

Procedure

1. About 50 mg of non-friable mass of callus was aseptically inoculated on semi solid medium having various PGR concentrations along with casein hydrolysate (50mg/l,100mg/l)
2. The cultures were incubated in growth room and maintained in both light and dark conditions.
3. The cultures were periodically checked for responses as well as color of callus was noted down.

Title No. 6: Initiation of Suspension Culture using friable mass of Callus

To initiate suspension culture using friable mass of callus in MS medium supplemented with casein hydrolysate (50mg/l, 100mg/l) along with individual NAA and TDZ.

Requirements

1. Media: MS liquid medium supplemeted with:

a) Casein hydrolysate (50mg/l, 100mg/l)

b) NAA (1mg/l, 2mg/l, 3mg/l)

c) TDZ (1mg/l, 2mg/l, 3mg/l)

2. Glassware, instruments and equipments.

Procedure

1. About 50 mg of friable mass of callus was used for initiation of suspension culture in MS medium.
2. The cultures were maintained on rotary shaker at 200 rpm.
3. The culture bottles were maintained in light as well as dark by covering with a black paper.
4. Cultures were observed after an interval of two weeks for dispersion of callus.

OBSERVATIONS, RESULTS AND DISCUSSION

The cultures maintained were observed every week for any changes in their structures and the obtained results were noted after 6-8 weeks.

Title No. 1: To study individual effect of PGR', auxin: NAA, cytokinin: TDZ on different floral parts of *Punica granatum* L.

Table 3: Effect of individual NAA (1mg/l, 2mg/l, 3mg/l) on floral parts of *Punica granatum* L. on MS medium.

MEDIA COMPOSITION (MS)	INCUBATION	PETALS EXPLANT (6 weeks)	ANTHER (6 weeks)	FILAMENT (6 weeks)
NAA (1mg/l)	LIGHT	Large mass of pink callus	Swelling of anther along with off white callus formed	Swelling of filament and development of off white callus
NAA (1mg/l)	DARK	No response	No response	No response
NAA (2mg/l)	LIGHT	Smaller mass of pink callus	No response	Swelling of filament in the beginning followed by pink callus formation from the ends towards the centre
NAA (2mg/l)	DARK	Green callus showing precautious germination	No response	No response
NAA (3mg/l)	LIGHT	Large mass of green and pink callus developed	No response	Pink and off-white callus developed from entire filament
CONTROL	LIGHT	No response	No response	No response
CONTROL	DARK	No response	No response	No response

Table 4: Effect of individual TDZ (1mg/l, 2mg/l, 3mg/l) on floral parts of *Punica granatum* L. on MS medium.

MEDIUM COMPOSITION (MS)	INCUBATION	PETAL (6 weeks)	ANTHER (6 weeks)	FILAMENT (6 weeks)
TDZ(1mg/l)	LIGHT	Green and pink callus, smaller in mass	No response	Dark brown callus
TDZ (1mg/l)	DARK	No response	No response	No response
TDZ (2mg/l)	LIGHT	Green callus, smaller in mass	Off white callus developed from anther	Green colored callus formed from entire filament
TDZ (2mg/l)	DARK	Precautious germination	No response	No response
TDZ (3mg/l)	LIGHT	Green callus, smaller in mass	No response	No response
TDZ (3mg/l)	DARK	No response	No response	No response
CONTROL	LIGHT	No response	No response	No response
CONTROL	DARK	No response	No response	No response

Table 5: Effect of individual Picloram on petals of *Punica granatum* L on MS medium.

MEDIA COMPOSITION (MS)	INCUBATION	PETAL EXPLANT 4 weeks
Picloram (0.5mg/l)	LIGHT	No response
Picloram (0.5mg/l)	DARK	No response
Picloram (1.0 mg/l)	LIGHT	Friable mass of callus obtained from petal explants
Picloram (1.0mg/l	DARK	No response
Picloram (2.0mg/l)	LIGHT	No response
Picloram (2.0mg/l)	DARK	No response

Table 6: Effect of individual 2,4-D on petals of *Punica granatum* L. using on MS medium.

MEDIUM COMPOSITION (MS)	INCUBATION	PETAL 4 weeks
MS + 2,4-D (0.5mg/l)	LIGHT	No response
MS + 2,4-D (0.5mg/l)	DARK	No response
MS + 2,4-D (1.0 mg/l)	LIGHT	Light brown friable mass of callus obtained
MS + 2,4-D (1.0mg/l)	DARK	Off white friable mass of callus obtained
MS + 2,4-D (2.0mg/l)	LIGHT	No response
MS + 2,4-D (2.0mg/l)	DARK	Off white callus with brown patches obtained

Title No. 2: To study combined effect of NAA and TDZ using various concentrations on the petals, anthers and filaments of *Punica granatum* L.

Table 7: Combined effect on floral parts using various concentrations of NAA and TDZ on MS medium.

MS MEDIA	INCUBATION	PETAL (4 weeks)	ANTHER (4 weeks)	FILAMENT (4 weeks)
CONTROL	LIGHT	Off white colour callus with green patches	Swelling of anthers but no response	No response
CONTROL	DARK	No response	No response	No response
NAA(1mg/l),TDZ(1mg/l)	LIGHT	Off white colour callus with green patches.	Swelling and development of small green callus	No response
NAA(1mg/l),TDZ(1mg/)	DARK	Small green callus formed	Swelling of anthers but no response	Swelling of filament but no response
NAA(1mg/l),TDZ(2mg/l)	LIGHT	No response	Swelling of anther but no response	Swelling of filament but no response
NAA(1mg/l),TDZ(2mg/)	DARK	No response	Green callus	Swelling of filament
NAA(1mg/l),TDZ(3mg/l)	LIGHT	Light brown callus formed	No response	No response
NAA(1mg/l),TDZ(3mg/l)	DARK	No response	Off-white callus	No response

NAA(2mg/l),TDZ(1mg/)	LIGHT	Green callus with pink patches	Swelling of anther but no response	Swelling of filament but no response
NAA(2mg/l),TDZ(1mg/)	DARK	Green callus	No response	No response
NAA(2mg/l),TDZ(2mg/)	LIGHT	White callus with pink patches.	Small light green callus formed from separated anther.	Swelling of filament but no response.
NAA(2mg/l),TDZ(2mg/)	DARK	Small green callus	Swelling of anther	Swelling of filament
NAA(2mg/l),TDZ(3mg/)	LIGHT	Green mass of callus showing generation of direct embryogenesis	Green callus formed from separated anthers.	Swelling of filament development of green callus
NAA(2mg/l),TDZ(3mg/)	DARK	No response	No response	No response
NAA(3mg/l),TDZ(1mg/)	LIGHT	Swelling of petal but green callus formed only on the margin	Swelling of anthers but no response	Swelling of filament but no response
NAA(3mg/l),TDZ(1mg/)	DARK	Small green callus	No response	No response
NAA(3mg/l),TDZ(2mg/)	LIGHT	Off-white callus	Off-white callus	No response
NAA(3mg/l),TDZ(2mg/)	DARK	No response	No response	No response
NAA(3mg/l),TDZ(3mg/)	LIGHT	Green callus at the margin	No response	Swelling of filament
NAA(3mg/l),TDZ(3mg/)	DARK	No response	Off white callus	Swelling of filament

Title No. 3: Comparative study of different Natural Extracts on Embryogenic Callus obtained from Individual Plant Growth Regulators.

Table 9: Effect of Abscissic acid on somatic embryos obtained from individual effect of NAA and TDZ on MS medium.
EFFECT OF ABCISIC ACID

MS MEDIUM	**ABA (1mg/l) 10 weeks**	**ABA(2mg/l) 10 weeks**	**ABA(3mg/l) 10 weeks**
NAA (0.5mg/l)	Petal callus: callus turned to dark brown hard mass	Petal callus: no changes	Petal callus: off-white friable callus
	Anther callus: callus turned dark brown	Anther callus: turned dark brown	Anther callus:callus turned dark brown
	Filament callus: dark brown callus formed	Filament callus: callus turned brown	Filament callus:callus turned dark brown with pink patches
TDZ(0.5mg/l)	Petal callus: light brown large callus	Petal callus: off-white hard callus	Petal callus: callus turned dark brown with green patches.
	Anther callus: hard dark brown callus	Anther callus: callus turned dark brown	Anther callus: callus turned dark brown
	Filament callus: dark brown callus with slight pink patches.	Filament callus: callus turned dark brown	Filament callus: callus turned dark brown

Table 10: Effect of Coconut water on somatic embryos obtained from individual effect of NAA and TDZ on MS medium.

EFFECT OF Coconut water (CW)

MS MEDIUM	CW (10%) 10 weeks	CW (15%) 10 weeks	CW (20%) 10 weeks
NAA(0.5mg/l)	Petal callus: green to dark brown hard callus with pink patches.	Petal callus: callus turned dark brown showing precautious germination.	Petal callus: light brown callus showing precautious germination.
	Anther callus: callus turned dark brown	Anther callus:callus turned dark brown	Anther callus: callus turned dark brown
	Filament callus: huge mass of off-white and light brown callus	Filament callus: callus turned dark brown	Filament callus:callus turned dark brown
TDZ(0.5mg/l)	Petal: light brown to off-white callus showing onset of secondary embryogenesis	Petal callus: dark brown callus showing green structures after 10 weeks of sub culturing	Petal callus: callus turned dark brown
	Anther callus: callus turned dark brown	Anther callus:callus turned dark brown	Anther callus:callus turned dark brown
	Filament callus: callus turned dark brown	Filament callus: callus turned dark brown	Filament callus: callus turned dark brown

Table 11: Effect of Casein hydrolsate on somatic embryos obtained from individual effect of NAA and TDZ on MS medium.

EFFECT OF Casein hydrolsate(CH)

MS MEDIUM	CH(20mg/l)	CH(25mg/l)	CH(30mg/l)
NAA(0.5mg/l)	Petal callus: dark brown	Petal callus: dark brown callus showing green structures after 10 weeks	Petal callus: off white callus
	Anther callus: light brown callus	Anther callus: brown callus turned green after 10 weeks of sub culturing.	Anther callus: dark brown
	Filament callus: off-white callus	Filament callus: dark brown callus	Filament callus: dark brown callus
TDZ(0.5mg/l)	Petal callus: callus turned dark brown with green patches.	Petal callus: callus turned to dark brown, off-white along with green structures, hard in texture.	Petal callus: green callus with brown patches
	Anther callus: off-white callus	Anther callus: dark brown callus	Anther callus: off-white hard callus showing onset of secondary embryogenesis
	Filament callus: turned dark brown	Filament callus: turned dark brown	Filament callus: turned dark brown

Title No. 4: Effect Of Coconut water, Activated Charcoal and adenine sulphate on Embryogenic Callus.

Table 12: Effect of individual Adenine sulphate and Coconut water as well as in combination with activated charcoal, on embryogenic callus obtained from combined PGR's on MS Medium.

Medium	PETAL	ANTHER	FILAMENT
MS+CW (15%)	Green callus showing off-white patches.	Green callus showing somatic embryos	Small green callus with no much change
MS+CW (15%) + activated charcoal (2%)	Off-white callus showing precocious germination.	Off-white friable mass of callus	Off-white small callus
MS+Adenine sulphate (4mg/l)	Green callus showing light brown patches	Green friable mass of callus.	Off-white mass of non-friable callus showing somatic embryo.
MS+Adenine sulphate (4mg/l) + activated charcoal (2%)	Green non friable mass of callus showing pink patches	Green friable mass of callus	Off-white non friable mass of callus
MS+Activated charcoal (2%)	Green callus with slight increase in mass of callus	Green mass of callus	Green callus with pink patches.

Title No. 5: Maintenance of Callus on Semi-solid Medium.

Table 13: Individual effect of NAA and TDZ along with Casein hydrolysate(CH) on maintenance of non-friable mass of callus on MS medium.

MS medium	INCUBATION	Concentration of CH (mg/l)	OBSERVATION 10 weeks
CONTROL	LIGHT	-	No changes
CONTROL	DARK	-	Slight increase in mass of callus
TDZ(1mg/l)	LIGHT	50mg/l	No changes
TDZ(1mg/l)	DARK	50mg/l	Twice increase in mass of callus
TDZ(1mg/l)	LIGHT	100mg/l	No changes
TDZ(1mg/l)	DARK	100mg/l	Slight increase in mass of callus
TDZ(2mg/l)	LIGHT	50mg/l	No changes
TDZ(2mg/l)	DARK	50mg/l	Thrice increase in mass of callus, whitish callus formed
TDZ(2mg/l)	LIGHT	100mg/l	Thrice increase in mass of callus
TDZ(2mg/l)	DARK	100mg/l	No changes
TDZ(3mg/l)	LIGHT	50mg/l	No changes
TDZ(3mg/l)	DARK	50mg/l	No changes
TDZ(3mg/l)	LIGHT	100mg/l	No changes
TDZ(3mg/l)	DARK	100mg/l	No changes
NAA(1mg/l)	LIGHT	50mg/l	Eight times increase in mass of

			callus, light brown offwhite callus
NAA(1mg/l)	DARK	50mg/l	Twice increase in mass of callus
NAA(1mg/l)	LIGHT	100mg/l	No changes
NAA(1mg/l)	DARK	100mg/l	Slight increase in mass of callus, dark brown callus
NAA(2mg/l)	LIGHT	50mg/l	No changes
NAA(2mg/l)	DARK	50mg/l	Blackening of callus
NAA(2mg/l)	LIGHT	100mg/l	Slight increase of callus
NAA(2mg/l)	DARK	100mg/l	Slight increase of callus
NAA(3mg/l)	LIGHT	50mg/l	No changes
NAA(3mg/l)	DARK	50mg/l	No changes
NAA(3mg/l)	LIGHT	100mg/l	Slight increase of callus mass
NAA(3mg/l)	DARK	100mg/l	Slight increase of callus

Title No. 6: Initiation of Suspension Culture using friable mass of Callus.

Table 14: Initiation of suspension culture through individual effect of NAA and TDZ in dark and light conditions.

Ms media	Incubation	Ch (mg/l)	Obseravtion After 8 Weeks
TDZ(1mg/l)	LIGHT	50	No dispersion of callus
		100	Callus turned dark brown
TDZ(1mg/l)	DARK	50	separation of callus was not observed
		100	No callus separation
TDZ(2mg/l)	LIGHT	50	Blackening of callus
		100	No dispersion of callus
TDZ(2mg/l)	DARK	50	Browning of callus
		100	No callus separation
TDZ(3mg/l)	LIGHT	50	No dispersion of callus
		100	Separation of callus was not observed
TDZ(3mg/l)	DARK	50	No dispersion of callus
		100	No callus separation
NAA(1mg/l)	LIGHT	50	Dispersion of callus along with formation of green structures.
		100	No separation of callus
NAA(1mg/l)	DARK	50	Formation of dark brown green structures
		100	No dispersion of callus
NAA(2mg/l)	LIGHT	50	Dispersion of callus and formation of large number of green dark brown structures.
		100	No separation of callus
NAA(2mg/l)	DARK	50	Blackening of callus
		100	No dispersion of callus
NAA(3mg/l)	LIGHT	50	Callus turned black of callus
		100	No dispersion of callus
NAA(3mg/l)	DARK	50	Browning of callus of callus
		100	No dispersion of callus

DISCUSSION

The present study undertaken was mainly focused on standardization of regeneration protocol using petal as an explants for the purpose of direct gene transformation using 'gene gun method' for plant improvement.

There has been tissue culture studies aimed at micropropagation of *Punica granatum*.L. 'bhagva variety' through nodal segment culture, hypocotyls culture for direct shoot formation. Also anther culture and leaf culture have been undertaken for the same purpose in earlier studies. However no reports have been found regarding tissue culture of petals and filament for the purpose of regeneration, although anther culture has also been undertaken.

In the attempt to regenerate entire plant by exploting the totipotency of different explants of the pomegranate varieties different growth regulators have been used however this is the first attempt of using TDZ as a cytokinin and using it along with auxins to study responses of floral parts.

Initiation of suspension culture as well as further sub culturing of the embryos obtained is all directed towards plant regeneration protocol through single cell culture or somatic embryo culture for its mass multiplication through the use of bioreactor technology.

CONCLUSION

The results of this dissertation work showed that even at low salt concentration, a good response from the floral parts of *Punica granatum* was seen. Individual effect of auxin (NAA) on petal explants gave callus induction in all the cases. Petals placed on ¾th strength MS media lead to the formation of friable mass of callus. Positive results were obtained in medium concentrations of Picloram in light condition whereas, 2, 4-D gave positive results with medium and higher concentration in both light as well as dark conditions.

Individual and combined effect of NAA and TDZ on floral parts *of Punicagranatum* (petals, anthers and filaments) gave different levels of responses in light and dark conditions along with the color and texture of callus induced. Ascorbic acid at the concentration of 25mg /L against phenolic substances released leading to blackening of the media which was more prominent in petals kept under light conditions. NAA (1mg/l) gave good results in all the three explants, the prominent character of the callus induced being green mass with pink patches and heterogeneous, multicellular cells containing starch grains revealed through

cytology studies. TDZ (2mg/l) under light condition gave good results in all the three explants, the callus induced being green in color with off-white patches and the cytology studies showed a more homogenous mass of cells with distinct nucleus. Anthers associated with filaments gave better results as compared to the separated anthers. Precocious germination was seen in petals placed on media containing TDZ (2mg/l) and NAA (2mg/l) under light and dark condition respectively. The precociously germinated embryos had firm attachment with the callus mass and hence were inseparable.

As compared to the individual effect of NAA and TDZ, a better response was observed through their combined effect at various concentrations on anthers and filaments. Whereas comparatively less responses were obtained in petal explants. Also, the responses were observed within four weeks of initiation which was less as compared to the responses generated by the individual concentrations which took almost 6 weeks. NAA (2mg/l) and TDZ (3mg/l) under light condition gave best results in all the three explants leading to swelling of the explants followed by the formation of green colored callus. Direct somatic embryogenesis was obtained in petal explant whose cytology studies showed its firm attachment to the mother tissues confirming absence of callus formation. Abnormal embryo formation was also seen through the cytology study of the anther callus due to unfavorable conditions whereas heterogeneous mass of cells with distinct nucleus was observed through cytology studies of filament callus.

The study of different natural extracts on embryogenic callus showed good results in media containing coconut water along with 0.5 mg/l TDZ each leading to formation of green to off-white mass of callus along with precocious germination (Coconut Water, 15%). Whereas, in the case of Casein Hydrolysate (25mg/l, NAA 0.5mg/l), the callus turned dark brown with no changes until 8 weeks but formation of somatic embryos towards the end of 10 weeks.

Embryogenic callus on media containing Coconut Water, Adenine sulphate and activated charcoal individually and in combination was successful in maintaining its fresh green nature without undergoing secondary embryogenesis. Precocious germination was observed in media containing coconut water (15%) and activated charcoal (2%) in petals. Somatic embryogenesis was observed in filaments placed on Adenine sulphate (4mg/l).

NAA (1mg/l), Casein Hydrolysate (50mg/l) under light condition was favorable for maintenance of non-friable brown mass of callus which grew to almost twelve times as

compared to its initial mass during inoculation (i.e. 50mg) along with the formation of small friable callus.50 mg/l of Casein Hydrolysate in dark condition in combination with TDZ 1mg/l and 2mg/l showed eight times and five times increase in mass of callus respectively. TD (2mg/l) along with Casein Hydrolysate (100 mg /L) gave ten times increase in mass of callus under light condition.

In the attempt of standardization of media for suspension culture using individual PGR's under light and dark condition using non friable mass of callus. Liquid media containing NAA (1mg/l) and Casein Hydrolysate (50mg /L) placed under light condition gave large mass of somatic embryos whereas comparatively lesser mass of somatic embryos were obtained from NAA (1mg/l) and NAA(2mg/l) both containing 100 mg/l Casein Hydrolysate placed under light condition. The somatic embryos were further sub cultured using the technique of immobilization and direct inoculation. The later one gave faster response (3 weeks) on media containing 20% Coconut Water and 50 mg/l Casein Hydrolysate, but a more organized structure was seen from the encapsulated somatic embryos in media containing 20% Coconut water along with 0.5mg/l NAA and 0.5mg/l TDZ.

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