TISSUE CULTURE AND TRANSFORMATION STUDIES IN INDIAN CULTIVARS OF PAPAYA

(*Carica papaya L.*)

JAYEETA BANERJEE (BHATTACHARYA)

MAY 2002

TISSUE CULTURE AND TRANSFORMATION STUDIES IN INDIAN CULTIVARS OF PAPAYA (*Carica papaya L.*)

A THESIS

SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR

THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN **BOTANY**

BY

JAYEETA BANERJEE (BHATTACHARYA), M. Sc

PLANT TISSUE CULTURE DIVISION
NATIONAL CHEMICAL LABORATORY
PUNE – 411 008, INDIA

MAY 2002

Dedicated

to my

Parents

CONTENTS

ACKNOWLEDGEMENT

I would like to express my deep sense of gratitude to Dr. S. S. Khuspe, my research supervisor, Plant Tissue Culture Division, National Chemical Laboratory, for his guidance, constant encouragement, valuable advises and the freedom of work he provided all throughout my research work.

I am grateful to Dr. S. K. Rawal, for his support, help, valuable suggestions, patient listening, useful discussions during my research period as well as in writing of this thesis.

Thanks are also due to Dr. K.V.Krishnamurthy, Dr. B.M. Khan, Dr. (Mrs.) S. R. Thengane, Dr. Mrs S. Hazra, Dr. D.C. Agrawal, Mrs. Urmil Mehta Dr. Mrs N. Shahastrabudhhe and Dr. M. M. Jana for their timely help.

I am thankful to Dr. G.A. Salunke, NARP, Ganeshkhind (Mahatma Phule Krishi Vidyapeeth, Rahuri), for kindly providing me the papaya seeds used for this research work. Thanks are also due to Dr. M.G. Shitole, Head of Department and Dr. Shirke, of Botany department, Pune university, for their timely help.

It's my pleasure to offer my thanks to all the staff and non staff members of the PTC Division, my friends and colleagues, Swati, Mohan, Satish, Lata, Madhumita, Ramchander, Noel, Neelima, Rohini, Shaila and Sucheta for maintaining a pleasant working atmosphere and offering timely help. My special thanks are also due to Adityada, Rahul, Ranchander, Mr. Parag, Mr. Kale and Mr. Dinesh.

I am personally thankful to all those known and unknown faces who directly or indirectly helped me during the phase of work towards my thesis dissertation.

Last but not the least, it is difficult to word my gratitude towards my family members for their encouragement and support during this period. I am grateful to my parents for supporting me in every possible ways as and when I needed them.

I am sincerely thankful to my husband Dr. Anjan Banerjee without whose help and support it was just impossible to complete the mission.

Finally I would like to thank Dr. Paul Ratnasamy, Director, National Chemical Laboratory, for allowing me to submit my work in the form of a thesis. The financial support in the form of research fellowship by C.S.I.R, New Delhi, is duly acknowledged.

Jayeeta Banerjee (Bhattacharya*)*

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled " **Tissue culture and transformation studies in Indian cultivars of papaya (*Carica papaya* L.)** submitted by Jayeeta Banerjee (Bhattacharya) was carried out by the candidate under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

(Dr. S. S. Khuspe)
Research Guide

Pune

Date:

Key to abbreviations

^0C	Degree Celsius
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
ANOVA	Analysis of variance
B5	Gamborg's medium (1968)
BAP	6-Benzyl amino purine
bp	Base pairs
CaMV	Cauliflower mosaic virus
CTAB	Cetyltrimethylammonium bromide
cv.	Cultivars
Dicamba	3,6 Dichloro-2-methoxybenzoic acid
DNA	Deoxy ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
GA_3	Gibberellic acid
IBA	Indole Butyric acid
IPTG	Isopropyl-β-D-galactoside
Kb	Kilobases
KDa	Kilodaltons
KIN	Kinetin (6-furfuryl amino purine)
LD_{50}	Lethal dose 50
MB5	MS basal media+B5 vitamins
MOPS	3-(N-morpholino) propanesulphonic acid
MS	Murashige and Skoog medium (1962)
NAA	α-Naphthaleneacetic acid
PCR	Polymerase chain reaction
Picloram	4 amino-3,4,6-trichloropicolinic acid
SDS	Sodium dodecyl sulphate
TDZ	Thidiazuron (1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea
UV	Ultraviolet (light)
Vol/Vol	Volume/volume (concentration)
Wt/Vol	Weight/ volume (concentration)
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
Zeatin	6-(4-hydroxy-3-methyl-2-butenulamino) purine

SYNOPSIS

Carica papaya L. (2n = 18), a native of the tropical Americas, belongs to the small dicotyledonous family *Caricaceae*. It is an important fruit crop grown in tropical and subtropical countries of the world. This fruit crop is of major economic importance to India, which is one of the leading papaya producing countries of the world. Papaya is susceptible to a number of diseases. Among these, the aphid transmitted, Papaya Ring spot Virus (PRSV) is the most devastating. This disease severely affects papaya productivity. Recently, major efforts have been directed towards the introduction of new agricultural traits into papaya through genetic engineering to solve this problem. However, majority of the published reports on papaya pertains to exotic cultivars, which are not grown in India. Since, India is one of the major papaya producers, concerted efforts are being made to develop transgenic papaya varieties of local Indian cultivars. A high frequency, efficient plant regeneration system is a major prerequisite in this regard.

The present work entitled **"Tissue culture and transformation studies in Indian cultivars of papaya (*Carica papaya* L.)"** was undertaken with an objective to develop an efficient and reproducible *in vitro* plant regeneration protocol for papaya to fulfill the prerequisite of genetic transformation for the commonly grown Indian cultivars. Another objective of the work was to genetically transform papaya using *Agrobacterium tumefaciens*.

The present research endeavor has been divided into six chapters, followed by a summary, and a list of references and author's publications.

CHAPTER 1: GENERAL INTRODUCTION

This chapter covers the introduction of the genus papaya (*Carica papaya* L) and a thorough literature survey on *in vitro* regeneration and genetic transformation studies in papaya. In addition, papaya production in different states in India, area under cultivation has also been mentioned in this chapter. Objectives and aims of the present work are also outlined in this chapter.

CHAPTER 2: MATERIALS AND METHODS

Different methodologies employed in entire tissue culture work, histology and *Agrobacterium tumefaciens* mediated genetic transformation are described in this chapter.

Experiments towards isolation and cloning of the coat protein (Cp) gene during the study have also been partly described in this chapter.

CHAPTER 3: COMPARATIVE STUDIES ON *IN VITRO* AND *IN VIVO* SEED GERMINATION OF PAPAYA

A Comparative analysis between *in vitro* and *in vivo* seed germination has been described in this chapter. This chapter also describes the influence of various pre-sowing treatments, growth regulators, temperature and antibiotics on high frequency papaya seed germination.

CHAPTER 4: INDUCTION OF SOMATIC EMBRYOGENESIS AND PLANT REGENERATION IN PAPAYA.

The effect of different phytohormones either alone or in combination on induction of somatic embryos from immature and mature embryo axis explants of three different cultivars has been described in this chapter. A protocol of plant regeneration *via* somatic embryogenesis has been described here. Maturation, germination and acclimatization of the plants in soil have also been discussed. Studies on histology and SEM (Scanning Electron Microscopy) of somatic embryos are also described in this chapter.

CHAPTER 5: *IN VITRO* INDUCTION OF MULTIPLE SHOOTS AND PLANT REGENERATION FROM EMBRYO AXIS AND SHOOT TIP EXPLANT OF PAPAYA

Establishment of a plant regeneration protocol *via* multiple shoot induction from immature zygotic embryo and shoot tip explant of papaya has been described in this chapter. The influence of phytohormones and other culture conditions in induction and proliferation of multiple shoots from immature zygotic embryo and shoot tip explants are also described. In addition, the conditions for elongation, *in vitro* rooting and hardening of plantlets are also mentioned in this chapter.

CHAPTER 6: TRANSFORMATION STUDIES IN PAPAYA:

A: *Agrobacterium tumefaciens* mediated transformation in embryo axis explants

Conditions of *Agrobacterium tumefaciens* experiment affecting GUS gene expression have been described in this chapter. The integration of GUS (β-glucuronidase) gene in callus tissue has been confirmed by histochemical assay and histological study.

B: Isolation and cloning of the papaya ring spot virus coat protein gene:

Isolation of the papaya ring spot virus lesions from the fruit rind and its enrichment by infection of *Cucurbita* as the secondary host is described. Further isolation and purification of total and poly (A) rich RNA from the infected leaves of the *Cucurbita* plant is described. RT-PCR performed with the template RNA for the amplification of the PRSV coat protein gene and its cloning are described.

SUMMARY:

This part of the thesis summarizes the main findings of the **entire** work and its future applications.

KEY REFERENCES

1. Fitch, M.M.M., Manshardt R.M., Gonsalves D., and J.L. Slightom (1993) Transgenic papaya plants from *Agrobacterium*-mediated transformation of somatic embryos. Plant Cell Rep. 12 (5): 245-9.

2. Fitch, M.M.M., Manshardt R.M., Gonsalves D., Slightom J.L., and J.C. Sanford (1992) Virus resistant Papaya plants derived from tissues bombarded with the coat protein gene of papaya ringspot virus. Bio/Tech. 10: 1466-1472.

3. Litz, R.E., and R.A. Conover (1982) *In vitro* somatic embryogenesis and plant regeneration from *Carica papaya* ovular callus. Plant Sci Lett. 26: 153-158.

RESEARCH WORK PUBLISHED

1. **Bhattacharya J**. and S.S.Khuspe (2001) *In vitro* and *in vivo* germination of papaya (*Carica papaya* L.) seeds. **Scientia Horticulturae** 91: 39-49.
2. **Bhattacharya J**., S.S.Khuspe, N.N Renukdas and S.K.Rawal (2002) Somatic embryogenesis and plant regeneration from immature embryo explant of papaya cv. Washington and Honey dew (**Indian Journal of Experimental Biology (in press**).
3. **Bhattacharya J.** and S.S.Khuspe (2001) 2,4,5-T induced somatic embryogenesis in papaya. **Journal of applied horticulture (**accepted for publication).
4. **Bhattacharya J.,** S.S.Khuspe, N.N Renukdas and S.K.Rawal (2002). Multiple shoot induction and plant regeneration from immature embryo axes of papaya. (Manuscript communicated).

PAPER PRESENTED IN INTERNATIONAL CONFERENCES / SYMPOSIA

1. **Bhattacharya J.,** N.N. Renukdas and S.S.Khuspe **(2001)** Multiple shoot formation and plant let regeneration from immature embryo axis of papaya **(***Carica papaya* L.). In:

2nd International symposium of biotechnology of tropical and subtropical species, Institute of Botany, Academia Sinica, Taipei, Taiwan, 5-9 November.

2. **Bhattacharya J.** and S.S.Khuspe **(2001)** High frequency embryo induction in Papaya (*Carica papaya* L.) using 2,4,5-T. In: 4th International Plant Tissue Culture Conference at Dhaka, Bangladesh 1-3 November, 2001.
3. S.S.Khuspe, ML Mohan, S.K.Rawal and **J. Bhattacharya (2000):** Isolation and expression of PRSV (Papaya Ringspot Virus) coat protein gene. In: 3rd International Crop Science Congress 2000, Hamburgh, Germany, 17-22nd August.
4. **Bhattacharya J.** and S.S.Khuspe **(1999)** Direct somatic embryogenesis from immature zygotic embryos of papaya (*Carica papaya* L.). In: International Conference on Life Sciences in the next millenium, School of Life Sciences, University of Hyderabad, India, Dec 11-14.

PAPER PRESENTED IN NATIONAL CONFERENCES / SYMPOSIA

1. **Bhattacharya J.** and S.S.Khuspe (2001) Proliferative somatic embryogenesis from zygotic embryos of papaya (Carica papaya L.). In: 3rd National symposium on biochemical engn and Biotechnology, Biohorizon 2001,IIT, Delhi,23rd-24th February.
2. **Bhattacharya J.** and S.S.Khuspe (2001) 2,4,5-T induced somatic embryogenesis in papaya presented at 88th Indian Science Congress held in Delhi. IARI, Delhi from January 3-7
3. **Bhattacharya J**. and S.S.Khuspe (2000) Effect of seed type and growth regulators on in vitro seed germination of papaya (*Carica papaya* L.) seeds. Basic and applied aspect of Plant and microbial biotechnology5. Presented at Dept of Botany, Modern College, Feb 4-5th, 2000, Pune-5.
4. **Bhattacharya J.** and S.S.Khuspe (2000) *In vitro* regeneration of papaya through somatic embryogenesis. In: 87th Indian Science Congress held in Pune University, Pune from January 3 –7.

(Dr. S. S. Khuspe)
Research Guide

Jayeeta Banerjee (Bhattacharya)
Candidate

CHAPTER 1

GENERAL INTRODUCTION

1. Crop

Papaya (*Carica papaya* L.) is an important and a very popular fruit crop of the tropical and subtropical countries of the world. The crop has a high food value and is sought by all classes of people for its medicinal properties. Demand of the crop among consumers is high because of its early bearing habit and as it produces fruit throughout the year. It is a member of small dicotyledonous family *Caricaceae*. The *Caricaceae* consists of four genera: *Carica*, *Cylicomorpha*, *Jacaratia* and *Jarilla*, all of which are native of tropical America except for the *Cylicomorpha*, which is indigenous to Africa (Badillo 1967, 1971). The genus *Carica* contains about 21 species of which *C. papaya* (2n=18) has got the major economic importance (Litz 1986a) followed by *C. monoica*, *C. cauliflora*, *C. stipulata*, *C. pubescence* etc. However, *C. candamarcencis* Hook.f. *C. monoica* Desf., *C. erythrocarpa* Heilborn., babaco, *pentagona* Heilborn, *C. quercifolia* Benth and Hook and *C. goudotiana* Solms-Lauback are also edible but are mainly used as preserves (Purseglove 1968; Jordan *et al.*1982; Litz 1984). Papaya is grown all over tropics and subtropics regions. However, major papaya producing countries are Brazil, Nigeria, Congo, Indonesia, Malaysia and India. The total area under papaya cultivation in India is 57,000 Ha (FAO 2000).

1.1 Origin and Distribution

Though the exact area of origin is unknown, the papaya is believed to be originated from tropical America, perhaps in southern Mexico and Costa Rica (Purseglove 1968). The plant is closely related to *C. petlata*, which occurs in this area and may have arisen, by hybridization. It was taken by Spanish to Manila in the mid 16^{th} century and reached to Malacca shortly and India by 1596 (Knight 1980). Papaya has now spread to all tropical and subtropical countries.

1.2 Ecology

Papaya is a tropical evergreen plant and is grown in latitudes of 32 ^{0}N and S. It requires warm and humid climate and can be cultivated up to a height of 1000 meters above sea level. Papaya cultivation is restricted by frost. The temperature below 10 ^{0}C affects adversely on the growth and developments on fruits, which ultimately delays fruit maturity and ripening. At lower temperature the fruits cannot develop flavour and also causes poor taste. Dry climate characterized by a little rainfall tends to add to the

sweetness of fruit whereas wet climate with heavy rainfall tends to reduce sweetness. The optimum range of temperature for proper growth of papaya is between 22 ^{0} to 26 ^{0}C (Muthukrishnan and Irulappan 1987). Annual rainfall should range between 1500 and 1800 mm and should be suitably distributed. The plant needs protection against winds. For successful cultivation, papaya requires moderate temperature coupled with low humidity and adequate soil moisture. Being a shallow rooted crop, the plant can be grown in soils about 45 cm deep. Papaya thrives best in well drained fertile soil with pH of 6-6.5. It cannot withstand water logged condition and results in rotting of stem and yellowing and dropping of lower leaves. A well-drained sandy loam soil, rich in food is the best for papaya cultivation (Singh and Dahiya 1982). Papaya growing areas in the world, production and area under cultivation have been given in Table 1.1.

Table 1.1 Country wise production of papaya in the year 2000

Country	Area (Ha)	Production (Mt)
Brazil	40000	3300000
Nigeria	90000	748000
India	**57000**	**644000**
Mexico	23163	636119
Indonesia	34890	450009
Dem republic of Congo	13000	213000
China	5600	179443
Peru	13819	165000
Thailand	9800	119000
Venezuela	5709	89522
Equador	5700	88920
Philippines	6436	74713
Yemen	4131	67945
Colombia	2500	65000
Malaysia	56000	56000
Bangladesh	5665	40000
Cuba	1919	36519
Costa Rica	754	36290
Mozambique	3400	31000
Dominican Republic	5892	28268

Table 1.1 Continued….

Table 1.1 (Contd)

Country	Area (Ha)	Production (Mt)
South Africa	2600	26000
Bolivia	2140	22700
United States of America	770	19500
Guatemala	400	16000
Jamaica	640	13700
Paraguay	1000	12000
Samoa	640	10000
Pakistan	1000	8600
Australia	300	7000
Chile	370	6500
Elsalvador	300	3300
Oman	350	2900
Argentina	150	2100
Puerto Rico	114	1905
Guinea- Bissau	200	1600
Cook islands	50	908
Fiji Islands	25	800
Honduras	40	650
Reunion	35	420
French Guiana	60	314
Islamic Republic of Iran	19	100
Israel	5	50

Source: FAO 2000

1.3 Status of Papaya production in India

Fruits and vegetables are grown only on 6 - 7% of gross cropped area but contribute more than 18.8% of the gross value of agricultural output and 52% export earnings from total agricultural produce. During the last few years, considerable emphasis has been given to this sector. Accordingly, areas under fruit production has increased by 172% from 1961 - 1993 and productivity per hectare was nearly doubled, leading to an increase in production to the tune of 320% (Anonymous 2000).

At present, the total production of papaya in India is about 64,4000 Mt from a cultivated area of 57,000 hectares (FAO 2000). The crop is gown all over the country and is available round the year. Karnataka is the leading contributor in India's total output of papaya production. However, Orissa, Assam, West Bengal and Gujarat also provide ideal

climatic conditions for its growth and thus contribute significant share in total papaya production in India. The scenario of area under cultivation and papaya production, state wise for the year of 1998-1999 has been presented in Table 1.2, the last ten years scenery has been presented in Table 1.3 and India's country-wise papaya export has also been presented in Table 1.4.

Table 1.2 State wise papaya production in India (1998 -1999).

State	Area under cultivation (Ha)	Production (Tones)
Andhra Pradesh	1.4	105.8
Arunachal pradesh	0.6	2.6
Assam	7.3	108.5
Gujarat	3.9	161.4
Haryana	0.5	25.0
Himachal pradesh	0.2	1.0
Karnataka	5.7	496.8
Kerala	12.7	56.1
Madhya Pradesh	0.9	43.0
Maharashtra	5.8	46.6
Manipur	1.9	10.3
Meghalaya	0.5	4.0
Mizoram	0.3	3.2
Nagaland	0.3	2.6
Orissa	17.9	285.0
Rajasthan	0.5	15.9
Tripura	0.5	2.7
Uttar Pradesh (plain)	0.4	9.5
West Bengal	6.0	199.2
Andaman & Nicober islands	0.2	1.8
Lakshadweep	0.1	0.4
Pondicherry	0.1	1.0

Source: National Horticultural Board, Ministry of Agriculture, Govt. of India.

Table 1.3 Area and production of papaya in India

Year	Area (Ha)	Production (Metric tones)
1990	36518	451497
1991	36579	389908
1992	37543	424123
1993	40000	450000
1994	42000	470000
1995	45000	505079
1996	44000	422495
1997	50000	550000
1998	67700	1582000
1999	57000	644000
2000	57000	644000

Source: FAO 2001

Table 1.4 India's country-wise exports of fresh papaya in 1997-1998

Country	Quantity (Metric tones)	Value (Rs. Lakhs)
Baharain	55.40	7.29
Belgium	56.86	8.18
Kuwait	152.74	17.57
Malaysia	-	-
Qatar	56.38	6.62
Saudi Arabia	204.93	21.21
South Africa	34.00	9.81
UAE	226.94	25.95
UK	6.46	2.39
USA	1.60	0.16
Total*	826.65	104.02

*Including others

Source: India’s Directorate General of Commercial Intelligence and Statistics, DGCIS, 2001

Fig 1.1

Fig. 1.1

A. Healthy papaya tree (cv. Co-2) in bearing. Arrow indicates leaf scars

B. Top (crown) of the papaya tree (cv. Washington) showing the position of its fruits and leaves

Maharashtra, with a total area of 1763 Ha land under papaya cultivation and production of 25930 tons also contributes a good share in Indian papaya scenario. District Beed and Nanded ranks first in papaya production in the state with a yield of 50 tons/ha (Anonymous 2000).

1.4 Plant Habit

Commonly and erroneously referred to as a "tree", papayas are one of the fastest growing fruit crop. Flowers can be produced as early as 4 months after germination of seed (Fitch 1995**).** The papaya is a rapidly growing plant (sometimes referred as herb) with single straight hollow green or deep-purple stem becoming 30-40 cm or more thick at the base and roughened by leaf scars (Fig.1.1A). The leaves emerge directly from the upper part of the stem in a spiral on nearly horizontal petioles 30-105 cm long, hollow, succulent, green or more or less dark purple. The blade is deeply divided into 5 to 9 main segments, each again is irregularly subdivided which varies from 1 to 2 ft (30-60 cm) in width and has prominent yellowish ribs and veins (Fig. 1.1B). Leaf morphology changes during development from single lobed juvenile leaves to palmate leaves of mature plants (Litz 1986). The life of a leaf is about 4 to 6 months. Laticifers occur in all glands through out the plant (Purseglove 1968**).**

1.4.1 Flowers

In nature, these plants are dioecious or hermaphrodite. Male flowers are morphologically distinct from female flowers. Male inflorescence is borne in many-flowered panicles of cymes on horizontal or pendent stalks to 1 meter long (Fig.1.2 A). The flowers are yellowish, 2-4 cm long. The petals are fused into a long tube, have 10 fertile stamens, and a rudimentary, non-functional ovary. Female inflorescence is much shorter only 3-4 cm long and has fewer flowers. Female flowers are larger, usually white or cream in colour, with five free petals. There are no stamens, but a large ovary with 5 fan-shaped stigmas (Fig. 1.2B) is present. The flowers of female plants are usually single whereas the inflorescence of the male plants is cymose (Litz 1986).

In cultivation, there are many intermediate forms, including bisexual flowers. At least 15 different flower forms have been named (e.g. Pentandria 5 stamens and a

functional ovary) and, because they are correlated with different fruit characteristics, some forms have been selected by breeders. Environmental factors may

also influence sexual expression and the sexuality of a plant may change seasonally or over the course of its lifetime. Female plants do not undergo sex reversal; it is only the

Fig. 1.2

A. A male plant (cv. Co-2) in flowering

B. A female plant (cv. Co-2) showing the position of flowers

hermaphrodite and male trees, which undergo this reversal. The dioecious condition has developed from the hermaphrodite condition (Litz 1986). Thus, the sexuality of any one plant in cultivation depends on a complex mixture of genetic, developmental and environmental factors.

1.4.2 Fruit

Generally, the fruit is a fleshy berry, melon-like, oval to nearly round, somewhat pyriform, or elongated club-shaped, 6 to 20 inches (15-50 cm) long and 4 to 8 inches (10-

20 cm) thick; weighing up to 20 lbs (9 kg). Semi-wild (naturalized) plants bear miniature fruits 1 to 6 inches (2.5-15 cm) long. Fruits from bisexual plants are usually cylindrical or pyriform with small seed cavity and thick wall of firm flesh, which stands handling and shipping well. In contrast, fruits from female flowers are nearly round or oval and thin-walled. The skin is waxy and thin but fairly tough. When the fruit is green and hard, it is rich in white latex (Fig. 1.3A). As it ripens, it becomes light or deep yellow externally and the thick wall of succulent flesh becomes aromatic, yellow, orange or various shades of salmon or red (Fig. 1.3B). It is then juicy, sweetish and somewhat like a cantaloupe in flavour. Attached lightly to the wall by soft, white, fibrous tissue, are usually numerous small, black, ovoid, corrugated, peppery seeds about 3/16 inches (5 mm) long, each coated with a transparent, gelatinous aril.

1.5 Importance and Uses

1.5.1 Domestic use

The papaya has become an important fruit in the diet because of its cheap price and nutritious value (Table 1.5). It is regarded as a fair source of iron and calcium, a good source of vitamins A, B and G and an excellent source of vitamin C (ascorbic acid). Ripe papaya is a favourite breakfast and dessert fruit which is available year-round. It can be used to make fruit salads, refreshing drinks, jam, jelly, marmalade, candies and crystallized fruits.

Fig. 1.3

Fig. 1.3

A. An immature papaya fruit cut open

B. A mature papaya fruit cut open

Table 1.5 Food value per 100 g of edible portion

Component	Per 100 g edible portion
Food energy	59.0 KCal
Moisture	84.4%
Protein	1.0 g
Fat	0.1 g
Carbohydrate	13.5 g
Ash	0.5 g
Calcium	31.0 mg
Phosphorous	17.0 mg
Iron	1.0 mg
Sodium	2.0 mg
Potassium	337.0 mg
Beta-carotene	2431 µg
Vitamin B2	0.15 mg
Niacin	0.1 mg
Vitamin C	69.3 mg
Vitamin B1	0.08 mg
Magnesium	0.8 mg
Fibre	0.5 g

Source: Ministry of Agriculture Malaysia, 2001

Ripe papayas are most commonly eaten as fresh, merely peeled, seeded, cut in wedges and served with an half or quarter of lime or lemon. Sometimes a few seeds are left attached for those who enjoy their peppery flavour. The flesh is often cubed or shaped into balls and served in fruit salad or fruit cup. Firm-ripe papaya may be seasoned and baked for consumption as a vegetable. Ripe flesh is commonly made into sauce for shortcake or ice cream sundaes or is added to ice cream just before freezing or is cooked in pie, pickled, or preserved as marmalade or jam. Papaya and pineapple cubes, covered with sugar syrup and may be quick-frozen for later serving as dessert. Half-ripe fruits are sliced and crystallized as a sweetmeat.

Unripe papaya is never eaten raw because of its latex content. Even for use in salads, it must first be peeled, seeded and boiled until tender then chilled. Green papaya is frequently boiled and served as a vegetable. Cubed green papaya is cooked in mixed vegetable soup. Extract of papaya is a good source of Vit A, C and others (Table 1.5). Vitamin A is present in high concentrations in red fleshed fruits (Arriola *et al.* 1980). Young leaves are cooked and eaten like spinach in the East Indies and even used to

tenderize meat. Papaya leaves contain the bitter alkaloids, carpaine and pseudocarpaine, which act on the heart and respiration like digitalis, but are destroyed by heat.

Papaya seeds are used in some countries as a vermifuge, counter-irritant and abortifacient. Amino acids (18) identified in papaya seeds are principally, in descending order of abundance, glutamic acid, arginine, proline, and aspartic acid in the endosperm and proline, tyrosine, lysine, aspartic acid and glutamic acid in the sarcotesta. Flath and Forrey (1977) identified 106 volatile components in papaya.

1.5.2 Industrial uses

The latex of the papaya plant contains two proteolytic enzymes, papain and chymopapain. It has varied uses in the beverage, food and pharmaceutical industries, in chill-proofing beer, tenderizing meat, drug preparations for digestive ailments and treatment of gangrenous wounds. It is also used in bathing hides, degumming silk and softening wool. Because of its papain content, a piece of green papaya can be rubbed on a portion of tough meat to tenderize it. Sometimes a chunk of green papaya is cooked with meat for the same purpose. One of the best known uses of papain is in commercial products marketed as meat tenderizers, especially for home use. A modern development is the injection of papain into beef cattle a half-hour before slaughtering to tenderize more of the meat than would normally be tender. Papain-treated meat should never be eaten raw but should be cooked sufficiently to inactivate the enzyme. Papain has many other practical applications. It is used to clarify beer, also to treat wool and silk before dyeing, to de-hair hides before tanning, and it serves as an adjunct in rubber manufacturing (Morton 1977, Duke 1984). It is applied on tuna liver before extraction of the oil, which is thereby made richer in vitamins A and D. It also finds its use into toothpastes, cosmetics and detergents, as well as pharmaceutical preparations to aid digestion. Papain has been employed to treat ulcers, dissolve membranes in diphtheria, and reduce swelling, fever and adhesions after surgery. Papayas are used in fruit processing industry for making pickles, jam, jelly etc.

1.5.3 Medicinal uses

In tropical folk medicine, the fresh latex is smeared on boils, warts and freckles and given as a vermifuge. The unripe fruit is sometimes hazardously ingested to achieve abortion. Seeds, too, may bring on abortion. The root is ground to a paste with salt,

diluted with water and given as an enema to induce abortion. Crushed leaves wrapped around tough meat will tenderize it overnight. The leaf also functions as a vermifuge and as a primitive soap substitute in laundering. Dried leaves have been smoked to relieve asthma or as a tobacco substitute. Papaya latex is used as anthelmintic (dewormer) in livestock (Satrija *et al*. 1994) and also to cure dyspepsia and is externally applied in treatments of burns and scalds (Reed 1976**).** Apart from all these papaya also finds its use in treatment of cancer, constipation, kidney problems, intestinal disorders, ulcers and urology treatments to name a few in different countries all over the world. The fruits are also beneficial in piles, dyspepsia of liver, spleen and digestive disorders. Papaya has a number of anti microbial uses**.** Latex (with a minimum protein concentration of 138 µl/ml) and root extracts inhibit *Candida albicans*. However, aqueous extracts are not active. Extracts of pulp and seeds showed bacteriostatic properties when tested against *Staphylococcus aureua, Escherichia coli, Salmonella typhi, Bacillus subtilis*, and other bacteria *in vitro*.

1.5.4 Papaya allergy

It has been reported that the action of fresh papaya latex causes skin irritation during handling of the papayas while the pollen of papaya flowers causes asthma and rhinitis in sensitive individuals (Anonymous 2001).

1.6 Factors affecting papaya production

1.6.1 Abiotic factors

Papaya is very much susceptible to frost damage. Papaya plantations need to be protected against strong winds by providing effective windbreaks. As it is shallow rooted crop, it is highly sensitive to wet feet. Water stagnation even for a short time affects the yield whereas in extreme cases the plant may even die. Low temperature adversely affects the growth and yield of the crop resulting in delayed maturity and ripening. Growth is also hampered by dry climate. Dry spells causes abortion of floral and fruit structures leading to sterile phases of fruiting (Muthukrishnan and Irulappan 1987). However, dry climate characterized by little or meager rainfall tends to add to the sweetness of fruit whereas wet climate with heavy rainfall tends to reduce the sweetness.

1.6.2 Biotic factors

1.6.2.1 Pests

Following are some of the important pests attacking papaya.

Fruit fly- Among the various pests that cause damage to papaya production, a major hazard to papayas is the wasp-like papaya fruit fly, *Toxotrypana curvicauda* Gerstaecker. The female deposit eggs in the fruit, which will later be found, infested with the larvae. Only thick-fleshed fruits are safe from this enemy.

Papaya web worm- An important and widespread pest is the papaya webworm, or fruit cluster worm, *Homolapalpia dalera*, harbored between the main stem and the fruit and also between the fruits. It eats into the fruit and the stem and makes way for the entrance of anthracnose. Damage can be prevented if spraying of insecticides begun at the beginning of fruit set, or at least at the first sign of webs.

Aphids- Aphids (*Aphis gossypii* Glover and *Myzus persicae* Sulzer) are also becoming an important pest of papaya. Nymphs and adults inject their saliva and suck the plant sap. They are known to transmit mosaic and ringspot virus. The early symptoms of the disease are presence of necrotic spots on the leaves, which turn later on to blistered patches on green tissues. Destroying weeds and virus infected plant can check their infestations.

White fly- The tiny papaya whitefly, *Trialeuroides variabilis, is* a sucking insect and it coats the leaves with honeydew, which forms the basis for sooty mold development. Shaking young leaves will often reveal the presence of whiteflies. Spraying or dusting of insecticides should begin when many adults are noticed.

Red spider mite- Other pests requiring control measures the red spider mite, *Tetranychus seximaculatus* Riley, which sucks the juice from the leaves. In India, plant and fruit infestation by red spider has been a major problem. This pest along with the cucumber fly and fruit-spotting bugs feed on the very young fruits and cause them to drop. The broad mite damages young plants especially during cool weather.

Root-knot nematodes- *Meloidogyne incognita acrita* Chitwood and reniforin nematodes, *Rotylenchulus reniformis* Linford and Oliveira are detrimental to the growth and productivity of papaya plants and should be combated by pre-planting soil fumigation if the nematode population is high.

Apart from all these insects, birds, rats and bats are also found to cause damage to the papaya fruit production. Major insect pests and their scientific names are given in Table 1.6.

Table 1.6 Major insect pest of papaya

No	Insect pest	Scientific names
1	Fruit fly	*Toxotrypana curvicauda* Gerstaecker
2	Papaya web-worm	*Homolapalpia dalera*
3	Aphids	*Aphis gossypii* Glover and *Myzus persicae* Sulzer
4	White fly	*Trialeuroides variabilis*
5	Red spider mite	*Tetranychus seximaculatus* Riley
6	Root-knot nematodes and Reniforin nematodes	*Meloidogyne incognita acrita* Chitwood and *Rotylenchulus reniformis* Linford and Oliveira
7	Immature earthworms	*Megascolex insignis*

1.6.2.2 Diseases of papaya

Diseases are the most important limiting production problems of papaya. Although the plant is classified botanically as perennial, virus diseases have reduced the effective crop life to 1-2 years (Pernezny and Litz 1993). Following are some of the major diseases observed in papaya.

Papaya Ringspot Virus- Papaya Ringspot Virus (PRSV) occurs in most of the papaya producing countries and is the most harmful one (Purcifull *et al*. 1985). The virus is probably the single threat in commercialization of papaya cultivation (Fig. 1.4A). The disease has been named because of striking symptoms that appear on the fruit (Fig. 1.4B). These consist of circles and C-shaped markings that are darker green than the background fruit color. Later these markings become gray and crusty in texture. Earliest symptoms appear as yellowing and vein clearing of younger leaves. A prominent yellow mottling of the leaves follows. One or more lobes of infected leaves may become severely distorted and narrow (Fig. 1.5). Infected plants exhibit growth reduction. There is reduced fruit set and quality especially flavour is adversely affected. Papaya Ringspot Virus can be transmitted mechanically and by grafting. However, it is thought that aphid transmission is the most important mechanism for disease spread in the field. Attempts to reduce disease levels by applying aphicides have not been successful. Recently two transgenic

papaya lines showed resistance in the field trial in Hawaii against the disease (APS net feature 1998).

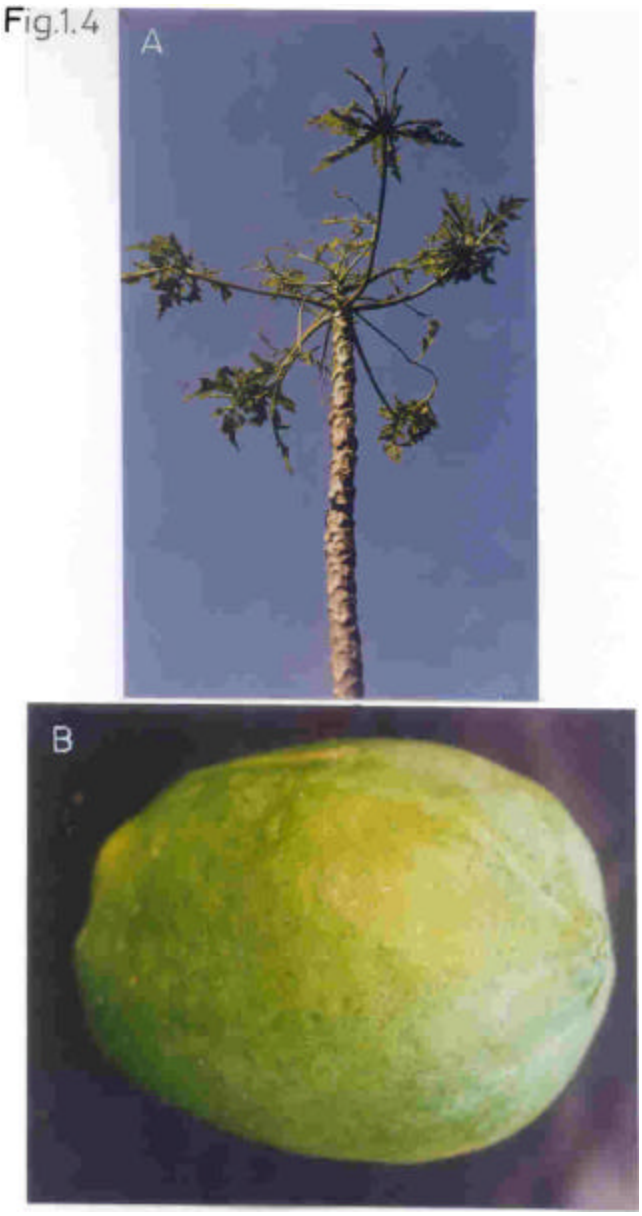


Fig. 1.4

A. A PRSV (Papaya Ringspot Virus) infected plant (cv. Washington) in field

B. An infected fruit (cv. Co-2) showing the characteristic ring spot in the fruit

Fig. 1.5

A. Papaya leaf infected with PRSV (Papaya Ringspot Virus)

Stem Rot (Fungus - *Pythium aphanidermatum* (Edson) Fitzp and *P. ultimum* Trow) -This disease was seen on young transplants. The initial symptom is a water soaking of the lower stem followed by its disintegration. Plants eventually wilt. Often, the white, cottony growth of the fungus is visible. Transplants should be planted in soil with good drainage. Stems should not be banked with soil.

Anthracnose (Fungus - *Colletotrichum gloeosporioides* Perz)- The initial symptoms are water soaked, sunken spots one-quarter to one inch in diameter on fruit. The centers of these spot later turns black and then pink when the fungus produces spores. The flesh beneath the spots becomes soft and watery, which spreads to the entire fruit. Small, irregular-shaped water soaked spots on leaves may also be seen. These spots eventually turn brown. Measures include removing the fruit as soon as it matures, removing all dead leaves and fruit from the vicinity of the plants, and removing infected fruits from the trees. Under conditions of severe disease pressure that would be found in more rainy,

humid climates, fungicides may be used. Allan (1976) indicated that the dioecious South African cultivar Honey Gold has some resistance to anthracnose.

Black Spot (Fungus - *Asperisporium caricae* (speg.) Maubl)- The initial symptoms are water-soaked spots on the upper leaf surface. Later, small, black spots are visible on the underside of the leaf. Black spots may also be found on the fruit. The tissue beneath them becomes corky, but the fruit does not rot. If there is a high incidence of infection of young leaves, a fungicide may be used.

Root rot (Fungus-*Pyhtium aphanidermatum* Matum Fitzp, *P. palmivora* E.J. Butler)- Because of the disease, young leaves wilt and mature leaf turn yellow and drop. It also causes root rot, collar rot and fruit rot. In case of severe infection, spraying with insecticides can be undertaken.

Bunchy top (MLO- Mycoplasma like organism)- Leaves and petioles show reduced growth, and become rigid. Internodes become progressively shortened, and petioles tend to assume a more horizontal position. Oily appearing spots are often present in the upper parts of stems and in petioles. Apical growth ultimately ceases, which, with the shortening of internodes, imparts a "bunchy top" appearance to affected plants. Bunchy top can be distinguished from boron deficiency by the fact that the tops of affected plants do not ooze latex when pricked.

Die-back- Papaya plants are subjected to die-back, a malady of unknown origin, which begins with shortening of the petioles and bunching of inner crown leaves. Then the larger crown leaves quickly turn yellow. Affected plants can be cut back at the first sign of the disease and if the cut stem is covered to avoid rotting, the top will be replaced by healthy side branches. The problem occurs mainly in the hot dry spring after a season of heavy rains.

Phytophthora blight (Fungus- *Phytophthora parasitica* Dastur)- A major disease in wet weather is phytophthora blight. *Phytophthora parasitica* attacks and rots the stem and roots of the plant and infects and spoils the fruit surface and the stem-end, inducing fruit fall and mummification. Fungicidal sprays and removal of diseased plants and fruits will reduce the incidence.

Powdery mildew (Fungus-*Oidium caricae F.* Noack*)*- This fungus often affects papaya plants. Sulfur, judiciously applied, is an effective control.

Damping-Off (Fungus - *Rhizoctonia solani* Kuhn)- This is a disease of young seedlings. Lesions are seen on the stem at or just above soil level. The stem becomes watery and shrinks, followed by death of the seedling. The diseases can be controlled using well drained soil and by avoiding over watering.

Other diseases

Cercospora leaf spot, or brown leaf spot, greasy spot or "papaya decline" (spotting of leaves and petioles and defoliation is caused by *Cercospora papayae* Hansf, which is controllable with fungicides. Names and type of the diseases and their causal organisms are given in Table 1.7

Papayas are frequently blemished by a condition called "freckles", of unknown origin; and mysterious hard lumps of varying size and form may be found in ripe fruits. Star spot (greyish-white, star-shaped superficial markings) appears on immature fruits after exposure to cold winter winds. In Uttar Pradesh (India), an alga, *Cephaleuros mycoidea,* often disfigures the fruit surface.

Table 1.7 Some common diseases affecting papaya

No	Disease	Type of organism	Name of causal organism
1	Damping-Off	Fungus	*Rhizoctonia solani* Kuhn
2	Papaya Ringspot Virus-	Virus	-
3	Stem Rot	Fungus	*Pythium aphanidermatum* (Edson) Fitzp and *P. ultimum* Trow
4	Anthracnose	Fungus	*Colletotrichum gloeosporioides* Perz
5	Black Spot	Fungus	*Asperisporium caricae* (speg.) Maubl
6	Root rot	Fungus	*Pyhtium aphanidermatum* Matum Fitzp, *P. palmivora* E.J. Butler
7	Bunchy top	MLO	Mycoplasma like organism
8	Phytophthora blight	Fungus	*Phytophthora parasitica* Dastur
9	Powdery mildew	Fungus	*Oidium caricae F.* Noack
10	Cercospora leaf spot	Fungus	*Cercospora papayae* Hansf
11	Die-back	Virus	-

1.7 Papaya Improvement

The improvement of papaya is hindered by its heterozygosity, dioecious habit and susceptibility to viruses. Clonally propagated selected genotypes would be of great value in breeding programs and in commercial propagation. Conventional grafting or multiplication by cutting is not possible in papaya. Tissue culture techniques may provide the only method to clonally propagate selected genotypes on a large scale (Bhasksran and Prabhudesai 1988).

1.7.1 Conventional propagation and Breeding

Papayas have been seed propagated despite the facts that uncontrolled pollinations can cause considerable and rapid genetic drift particularly in dioecious papaya cultivars (Litz 1986). Rouging unwanted male plants from dioecious papaya plantings and all female plants from planting of hermaphrodite papaya cultivars is necessary. Allan (1964) and Sookmark and Tai (1975) have described procedures for the asexual propagation of papaya by stem cuttings. However, this procedure is slow and inadequate for propagation of new or improved cultivars. The hermaphrodite varieties produce true to type seeds if pollinations are controlled, although segregation of sex types does occur (2 hermaphrodite: 1 female). The dioecious papaya cultivars are maintained by means of controlled pollination (sib mating), poly crossing and recurrent selection. Although papaya can be propagated by seed, if pollinations and seed productions are carefully controlled, it is inevitable that there will be continuos genetic drift among dioecious cultivars unless the parental lines are maintained indefinitely or if the cultivar itself can be cloned.

1.7.2 Need for non conventional methods for breeding and propagation

Diseases and environmental stresses are usually much more important factors limiting the food production in the tropics and subtropics than in temperate zones. The most serious threat to papaya production is the occurrence of Papaya Ringspot disease all over the world which hinders plant development, fruit yield and papain production. Resistance to these diseases is low or nonexistent. Monogenic resistance to Papaya Ringspot Virus occurs in three related species *C. pubescens ,C. stipulata and C. cauliflora* all of which are sexually incompatible to papaya. (Jimenez and Horovitz 1958, Horovitz and Jimenez 1967). Papayas cannot tolerate frost and a temperature near or below freezing point (Singh and Dahiya 1982), which effect sugar levels and quality of

papaya fruits. *Carica pubescens* are more cold tolerant than papaya and C. *candamarcensis* and *C. pentagona* are resistant to frost (Singh 1964). A need exists for an efficient method for embryo rescue following wide interspecific hybridization and possibly for controlled interspecific protoplast fusion to affect these crosses. This would extend the geographical range for papaya production and should also improve the quality of papaya fruits in areas with cool winter temperatures.

1.7.3 *In vitro* approaches

Several micropropagation systems have been developed for the papayas (Table 1.8). Unlike most tropical and subtropical fruit trees, papaya is a herbaceous plant, which probably has facilitated the development of *in vitro* approaches. Several organogenesis and somatic embryogenesis procedures have been developed. Among the *in vitro* approaches reported, plant regeneration *via* micro-propagation and somatic embryogenesis method have been developed in few exotic varieties which are not well adopted in Indian climatic condition. Techniques of anther culture, embryo rescue, protoplast culture have been developed which should have considerable effect on papaya improvement programs.

1.7.3.1 Embryo Culture

One of the goals of papaya improving scheme is to hybridize the papaya with the sexually incompatible *Carica* species particularly *C. cauliflora* Jacq. which is resistant to infection by papaya Ringspot Virus. In compatibility between papaya and *C. cauliflora* is caused by failure of endosperm formation. Phadnis *et al.* (1970) successfully cultured mature papaya embryos on Whites medium containing 0.24 µM KIN, 0.3 µM GA_3 and 0.6 µM IAA. De Bruijne *et al.* (1974) was successful in culturing mature papaya embryos on MS medium without meso inositol, casein hydrolysate or vitamins, but containing 58.4mM sucrose**.** Khuspe *et al.* (1980) demonstrated the practicality of using embryo culture to rescue immature embryos that resulted from *C. papaya* L. X *C.cauliflora* Jacq. crosses. Solid White's medium without vitamins but supplemented with 58.4mM sucrose, 2.0 µM cupric chloride and 1.2 µM sodium molybdate was used to germinate excised immature hybrid embryos in the dark. Subsequently the young plantlets were subcultured in liquid White's medium (1963) with vitamins and glycine where they were able to grow to transplantable size.

1.7.3.2 Shoot tip culture

Mehdi and Hogan (1976) established papaya seedling shoot tips on MS medium containing 4.7 µM KIN and rooted them on medium with 4.7 µM KIN and 24.5 µM IBA. They did not observe multiple shoot formation. Later Yie and Liaw (1977) could establish papaya seedling shoot tips *in vitro* and obtained proliferated growth on MS medium containing 0.3 µM IAA and either 23 µM KIN or 2.2-4.4 µM BA. Also Litz and Conover (1977, 1978 a) developed a procedure for establishment and culture of excised shoot tips from field grown mature papayas. They found that establishment time and rate of proliferation were both dependent on age of the stock plant, time of year, sex types and presence of bacterial contaminants (Litz and Conover 1981b). Staminate plants responded more rapidly than pistillate ones and had greater proliferation potential.

1.7.3.3 Organogenesis

There are several reports on organogenesis. Yie and liaw (1977) first observed adventitious shoots from callus in a culture medium containing MS basal media supplemented with 0.3 µM IAA and 4.7 µM KIN or with 9.4 µM KIN alone. Later, Arora and Singh (1978c) reported that transfer of papaya callus was necessary from a callus induction medium to a shoot induction medium where they could achieve roots from the callus, cultured in media supplemented with KIN and NAA. Formation of adventitious roots from midrib callus of papaya cotyledons was also achieved (Litz *et al.*1983) on MS medium with NAA and BAP. Also in the same media combination, adventitious meristems were formed from both lamina and midrib callus.

Table 1.8 Studies on micropropagation of papaya

No	Explant source		Reference
1	Seedling apices	Micropropagation. Few roots	Drew and Smith (1986)
2	Stem section and Shoot tips	Micropropagation. Few roots	Mehdi and Hogan (1976)
3	Six month old axillary rooted cuttings or buds	Micropropagation rooted plants to field	Drew (1988)
4	Six month old shoots	Micropropagation	Miller and Drew (1990)

		rooted plants to field	
5	Lateral shoot or mainstem bud	Micro propagation	Reuveni *et al.*(1990)
6	Shoot apices	Micropropagation	Litz and Conover (1977)
7	Shoot apices mature field grown	Micropropagation	Litz and Conover (1978a)
8	Shoot apices	Micropropagation	Litz and Conover (1978b)
9	Shoot apices, field nursery plants	Micropropagation	Winnar (1988)
10	Seedling stem petiole shoot	Micropropagation	Rajeevan and Pandey (1983)
11	Field grown lateral shoots	Micropropagation	Rajeevan and Pandey (1986)
12	Shoot buds	Micropropagation	Mondal *et al.* (1990)
13	Petioles	Micropropagation	Yang and Ye (1992)
14	Petioles	Micropropagation	Hossain *et al.* (1993)
15	Petioles	Micropropagation	Lai *et al.* (1998)

1.7.3.4 Somatic embryogenesis

Protocols for somatic embryo induction in papaya have been developed for a variety of reasons ranging from interest in methods for mass propagation Yie and Liaw (1977), Mehdi and Hogan (1979), Chen *et al.* (1987), Chen (1988 a, b) to a requirement for recipient tissues for gene transfer technology (Fitch and Mansherdt 1990; Fitch 1993). Ironically, somatic embryos were produced in cultures of papaya hybrid embryos, which resulted from crosses between PRSV resistant wild species and commercial papaya cultivars. These hybrid embryos were recovered by ovule or ovary cultures or embryo rescue (Khuspe *et al.* 1980; Litz and Conover 1981a, 1982, 1983; Moore and Litz 1984; Chen and Kuo 1988; Chen 1988a; Mansherdt and Wenslaff 1989 a, b and Chen *et al.*1991) techniques. Cultures of other *Carica* explants *C. stipulata* peduncles (Litz and Conover 1980), *C. pubescens* hypocotyl (Jordan *et al*. 1982), and *C. x heilbornii* nm. Pentagona ovules (Vega de Rojas and Kitto 1991) were also highly embryogenic. The earliest report of successful somatic embryogenesis in papaya was that of De Bruijne *et al.* (1974) who induced somatic embryos from petiole sections cultured on Murashige and Skoog (MS) (1962) and White (1963) media in a multistep protocol. They obtained somatic embryos but were not able to regenerate plants. Yie and Liaw (1977), Mehdi and Hogan (1979), Chen *et al.* (1987), Chen (1988 a, b) Yamamoto and Tabata (1989) used

seedlings grown *in vitro* as their source of explant for somatic embryo induction. However, green house grown seedlings were also used (Jordan *et al*. 1982). Using the somatic embryogenesis protocols of Litz and Conover (1981 b, 1982, 1983) and Mansherdt and Wenslaff (1989 a, b) as guidelines, Fitch and Mansherdt (1990) produced somatic embryos for transformation studies. All these reports have been summarized in Table 1.9.

Table 1.9 Studies on somatic embryogenesis of *Carica papaya*

No	Explant source	Result	Reference
1	Petiole	EC	De Bruijne *et al*. (1974)
2	Seedling stem internodes	SE, P	Yie and Liaw (1977)
3	Seedling shoot tips, mature shoots, internodes, seedling internodes	SE, P	Mehdi and Hogan (1976)
4	Seedling stem, cotyledon, root leaf shoot tip	SE, P from root callus	Chen *et al*. (1987); Chen (1988)
5	Seedling pieces	C	Yamamoto *et al*.(1986)
6	Seedling pieces	SE	Yamamoto and Tabata (1989)
7	Immature zygotic embryos	SE, P	Fitch and Mansherdt (1990).

C-Callus; EC-Embryogenic callus; SE- Somatic embryos; P-Plants

1.7.3.5 Anther culture

Litz and Conover (1978a) first used the technique of anther culture in papaya. Interesting results were obtained by culturing anthers from 16 to 20 mm flower buds in liquid MS medium supplemented with 87.6 mM sucrose 8.8 µM BA, 2.7 µm NAA, and 10 g/l charcoal following a pretreatment of 40 ^{0}C for 3 to 4 days. Only a small number of haploid plantlets have been recovered using these techniques. Chromosome counts verified that the regenerated plants have the haploid number (n=9). However, successful regeneration of plants by anther culture was also reported (Tsay 1985).

1.7.3.6 Protoplast Isolation and culture

Litz and Conover (1979) first reported the large scale isolation of papaya protoplasts. Limited callus induction but with a low plating efficiency has been achieved (Litz 1986a). However, plant regeneration from *Carica* protoplast was successfully obtained by Chen and Chen (1992**).**

1.8 Genetic transformation studies

Genetic transformation study in papaya was first reported by Pang and Sanford (1988) by *Agrobacterium* mediated gene transfer. However, they could not regenerate plants from the transformed tissues. Following their success, Fitch *et al*. (1990) showed that particle bombardment resulted in stable expression of chimeric genes coding for NPTII and GUS in papaya.

With further continuation of studies, they produced virus resistant papaya plants derived from tissues bombarded with CP gene of papaya Ring spot Virus (Fitch 1992). Many authors (Ye *et al*. 1991, Fitch *et al*. 1993, Yang *et al*.1996, Cabrera ponce *et al*. 1996) have also reported the development of transgenic papaya through *Agrobacterium* mediated transformation (Table 1.10). Also techniques for particle bombardment mediated transformation of papaya were carried out (Cabrera ponce *et al*. 1995; Mahon *et al*. 1996; Cai *et al*. 1999). Recently success in replicase mediated resistance against PRSV in papaya has also been reported (Chen *et al*. 2001).

Table 1.10 Studies on genetic transformation of *Carica papaya* L. by *Agrobacterium* and particle bombardment methods

No	Explant used	Method used	Reference
1	Leaf stem and petiole	*Agrobacterium*	Pang and Sanford (1988)
2	Immature zygotic embryo, hypocotyl, embryogenic calli	Particle bombardment method	Fitch *et al*. (1990)
3	Somatic embryos	*Agrobacterium*	Ye *et al*. (1991),
4	Immature zygotic embryo, hypocotyl, embryogenic calli	Particle bombardment method	Fitch *et al*. (1992).)
5	Hypocotyl	*Agrobacterium*	Fitch *et al*. (1993),
6	Immature zygotic embryos	Particle bombardment method	Cabrera ponce *et al*. (1995)
7	Leaf	*Agrobacterium*	Cabrera Ponce *et al*. (1996)
8	Petioles	*Agrobacterium*	Yang *et al*.(1996),
9	Immature zygotic embryo	*Agrobacterium*	Cheng *et al*. (1996)
10	Immature zygotic embryo	Particle bombardment method	Mahon *et al*. (1996)

11	Immature zygotic embryo,	Particle bombardment method	Cai *et al*. (1999).
12	Roots and hypocotyls	*Agrobacterium*	Chen *et al*. (2001)

1.9 Aims of thesis

At the time of initiation of this study, there were very few reports of plant regeneration with cultivars generally grown in India and majority of the work was done with Australian or Hawaiian varieties. Introduction of foreign varieties may not yield well here in our country because of lack of adaptability to new climate. Therefore, any improvement in Indian cultivars of papaya for their use under different climatic conditions in India needs extrapolation of work already done with Hawaiian or Australian varieties which is a major prerequisite. During the initiation of the present study, as the three cultivars namely Honey Dew, Co-2 and Washington were very popular, we selected these cultivars for our majority of the experiments.

The objectives of the thesis are, therefore, aimed at fulfilling these prerequisites so that Indian cultivars of papaya with agronomically desirable traits through biotechnological methods could be evolved.

1) To study aseptic *in vitro* seed germination of papaya and compare it with *in vivo* germination.
2) To study *in vitro* regeneration of papaya *via* somatic embryogenesis.
3) To develop *in vitro* papaya plant regeneration system *via* organogenesis.
4) To study *Agrobacterium* mediated genetic transformation in papaya and studies relating to isolation and cloning of PRSV gene.

CHAPTER 2

MATERIALS AND METHODS (General)

This chapter describes the techniques routinely followed in plant tissue culture work. The material and methods, which are specific to the particular experiment, have been dealt in details in the respective chapters. Techniques of genetic transformation by *Agrobacterium* method used in the present study have been described in chapter 6 of the thesis. In addition, the procedures followed for isolation of mRNA, PCR amplification, purifying the PCR product, transformation to *E.coli* and confirmation of the transformants have also been described in detail in chapter 6.

2.1 Glassware

Glassware used in all the experiments was procured from "Borosil", India. Test tubes (25 mm x 150 mm), glass bottles (70 mm x 125 mm), petri dishes (85 mm x 15 mm), conical flasks (100, 250, 500 and 1000 ml capacity) and pipettes (1, 2, 5, 10 and 25 ml capacity) were used during the course of study.

2.1.1 Preparation of Glassware

All the glasswares were cleaned by boiling in a saturated solution of Sodium bicarbonate for 1h followed by repeated washing in tap water. Thereafter, these were immersed in 30% nitric acid solution for 30 min and followed by repeated wash in tap water. Washed glassware was further rinsed with distilled water and then dried at room temperature (ambient temperature) or in an oven at 200 ^{0}C. Test tubes and flasks were plugged with absorbent cotton (Seasons Healthcare Ltd, Andhra Pradesh, India). Pipettes and petri dishes were wrapped in brown paper and then sterilized in autoclavable polypropylene bag. Autoclaving of the glassware and above items was carried out at 121 ^{0}C, 15 lb. psi for 1 h.

2.2 Plasticware

Plasticwares such as **s**terile disposable filter sterilization units and petri dishes (35 mm, 55 mm and 85 mm diameter) were procured from "Laxbro", India. Eppendorf tubes (1.5 ml and 2 ml capacity), microtips (0-200 µl and 200-1000 µl capacity) were also obtained from "Laxbro" and "Tarsons", India. Wide bore microtips (0-200 µl) were procured from "Sigma", USA.

2.3 Chemicals

Chemicals used in all the experiments of tissue culture study were of analytical grade and were obtained from " Qualigens", "S.D fine chemicals" or "Hi-media", India. Molecular biology chemicals were obtained from "Sigma Chemical Co.," USA. Growth regulators, antibiotics (except cefotaxime) were also obtained from "Sigma Chemical Co.", USA. Cefotaxime was procured from Russel India Ltd. Bombay, India. Sucrose, glucose, gelling agent and agar-agar were obtained from "Qualigens" and "Hi-Media". Bacto-Agar for microbial work was obtained from "DIFCO®" laboratories, USA. Coconut milk (Liquid endosperm) was collected from tender coconuts, obtained from local market and stored at -20^{0} C as aliquots of 100 ml in 250 ml Erlenmeyer flasks after filtering through Whatman No. 1 filter paper and autoclaving for 20 mints.

2.4 Preparation of culture media

Double distilled water was used for preparation of all the culture media**.** After addition of all macro- and micro-nutrients, vitamins, growth regulators and other necessary carbohydrate source like sucrose or glucose, the pH of the media was adjusted to 5.8 before autoclaving using 0.1N NaOH or HCl. Volume was made and gelling agent was added as per requirement. The medium was steamed to melt the gelling agent.

Melted medium was then dispensed into test tubes, flasks and thereafter sterilized by autoclaving at 121 ^{0}C at 15 lb psi for 20 min. Thermolabile growth regulators and antibiotics were filter sterilized through a Millipore membrane (0.22µm or 0.45µm pore size). These were added to autoclaved medium before dispensing. Compositions of several basal media including Murashige and Skoog's (MS) and Gamborg's (B5) macro, micro elements and vitamins used in the present study are given in Table 2.1, 2.2 and 2.3 respectively.

Table 2.1 Composition of macro-element salts (mg/l) in five tissue culture basal media

Macro-element	MS [a]	LS [b]	SH [c]	WH [d]	B5 [e]
KNO_3	1900	1900	2500	80	2500
NH_4NO_3	1650	1650	-	-	-
$CaCl_2.2H_2O$	440	440	200	-	150
$MgSO_4.7H_2O$	370	370	400	737	250
KH_2PO_4	170	170	-	-	-
$NaH_2PO_4.H_2O$	-	-	-	16.5	150
$(NH_4)_2\ SO_4$	-	-	-	-	134

Table 2.2 Composition of micro element salts (mg/l) in five tissue culture basal media

Micro-element	MS [a]	LS [b]	SH [c]	WH [d]	B5 [e]
$MnSO_4.\ 4H_2O$	22.3	22.3	-	6.65	-
$MnSO_4.\ H_2O$	-	-	10	-	10
$ZnSO_4.7H_2O$	8.6	8.6	1.0	2.67	2.0
H_3BO_3	6.2	6.2	5.0	1.5	3.0
KI	0.83	5.0	1.0	0.75	0.75
$CuSO_4.5H_2O$	0.025	0.025	0.2	0.001	0.025
$Na_2MoO_4.2H_2O$	0.25	0.25	0.1	-	0.25
$CoCl_2.6H_2O$	0.025	0.025	-	2.5	0.025
$FeSO_4.7H_2O$	27.8	27.8	15	-	27.8
$Na_2EDTA.2H_2O$	37.3	-	20	-	37.2
MoO_3	-	-	-	0.0001	-
$Fe\ (So_4)_3$	-	-	-	2.5	-

Table 2.3 Composition of organics (mg/l) in five tissue culture basal media

Organics	MS [a]	LS [b]	SH [c]	WH [d]	B5 [e]
Thiamine. HCl	0.1	0.4	5.0	0.1	10
Pyridoxine HCl	0.5	-	0.5	0.1	1.0
Nicotinic acid	0.5	-	5.0	0.5	1.0
Myo-inositiol	100	100	1000	-	100
Glycine	2.0	-	-	3.0	-

[a] Murashige and Skoog (1962); [b] Linsmaier and Skoog (1965); [c] Schenk and Hilderbrandt (1972); [d] White (1963); [e] Gamborg *et al.* (1968)

2.5 Collection of Plant material

Seeds (Fig.2.1) of papaya cultivars Pusa Majesty, Pusa Delicious, Pinky Flesh, Ex-15, Disco, Hybrid 781, Hybrid 786, Honey Dew, Washington and Pant papaya were obtained from Regional Fruit Research Station, Ganeshkhind, Pune Maharashtra, India. The cultivar Honey Dew was obtained from D. J. Damani & Sons, Shivajinagar, Pune. Seeds were procured in the month of July-August and planted in our campus field for using the mature and immature fruits throughout the year.

2.6 Preparation of plant material

2.6.1 Surface sterilization of fruits and seeds

Mature seeds were surface sterilized using liquid detergent, ethyl alcohol sodium hypochlorite as a surface sterilizing agent. The process of surface sterilization has been described in details in Chapter 3. Fruits after collecting from field were washed under running water and then cleared in mild liquid detergent solution for 10 min. Fruits were then taken inside the laminar flow and treated with 70 % ethyl alcohol for 30 sec followed by dipping in 4% sodium hypochlorite solution (NaOCl) for 10 min. When dried fruits were cut and immature seeds (Fig.2.2) were taken out to excise the embryo.

The surface sterilization procedure is almost same for seeds and fruits, only difference being the time given for ethyl alcohol and sodium hypochlorite treatment is 10 min instead of 10 sec for seeds.

2.6.2 *In vitro* germination of seeds

Different conditions like basal media, presoaking treatment of sterilized seeds, incubation on moistened condition in petridishes, illumination conditions were evaluated

for high frequency *in vitro* germination of seeds. Details of this have been described in chapter 3. The 20-day-old seedlings were used for preparation of various explants like leaf, cotyledon, hypocotyl, roots etc.

2.7 Inoculation

Aseptic explants derived from surface sterilized fruits or seeds were inoculated in the media in a Laminar air-flow cabinet (Microfilt, India). Excision of the explants was

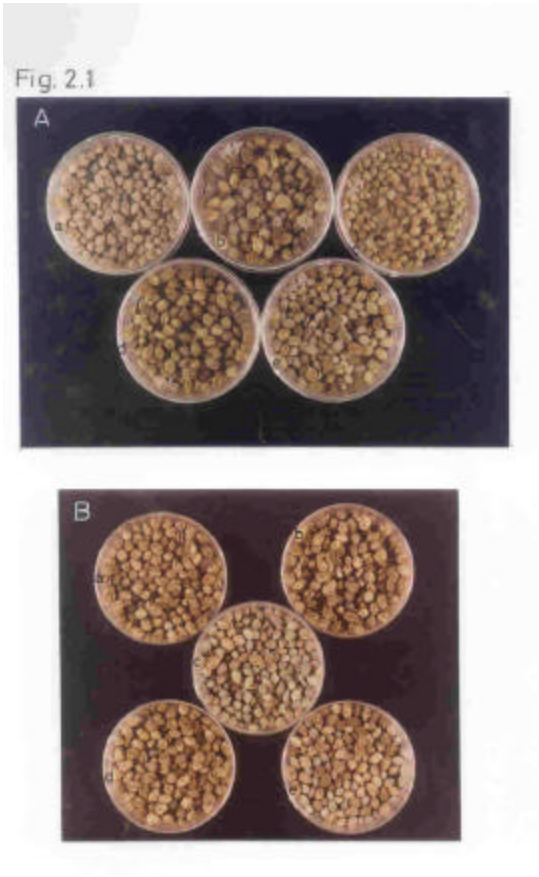


Fig. 2.1

A. Mature seeds of five cultivars, a-Pusa Majesty, b-Pusa Delicious, c-Pinky Flesh, d-Ex-15 and e-Disco

B. Mature seeds of five cultivars, a-Hybrid 781, b-Hybrid 786, c-Honey Dew, d-Washington and e-Pant papaya

carried out on sterile filter papers with the help of sterile scalpels and forceps. Scalpels and forceps were flame sterilized prior to inoculation and also in between the work by dipping in 70% rectified spirit. Surgical blades (No. 11) (Kehr Surgicals and allied products Pvt. Ltd, Kanpur, India) were used for excision of the explants. Sterile filter paper bridges (Whatman No.1) were used as supports for explants cultured in liquid

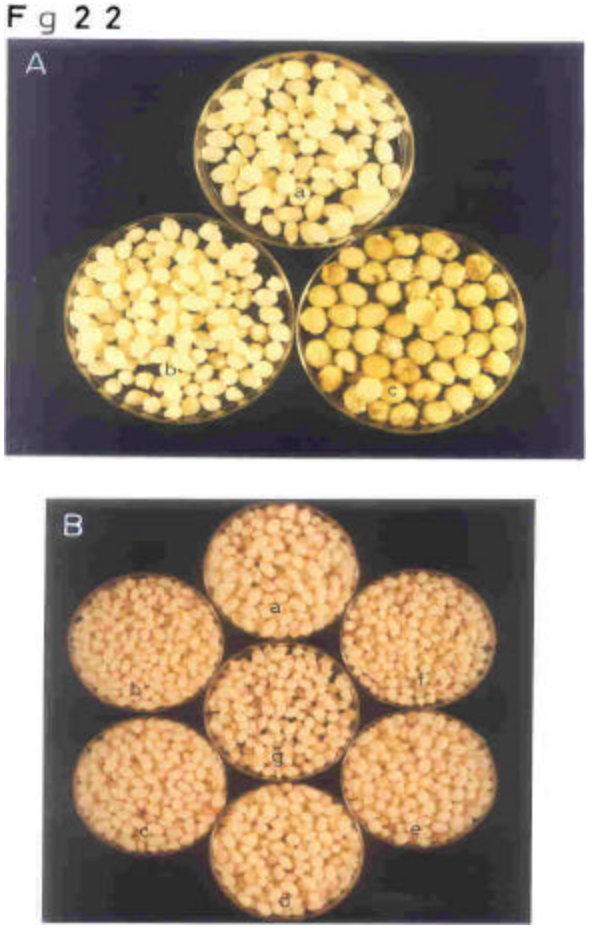


Fig. 2.2

A. Immature seeds of three cultivars, a- Pusa Majesty, b-Pusa Delicious, c-Pinky Flesh

B. Immature seeds of seven cultivars, a-Hybrid 781, b-Hybrid 786, c-Ex-15, d-Disco, e-Honey Dew, f-Washington and g-Pant papaya

media under static conditions. All the experiments were repeated thrice or otherwise mentioned. The number of explants and replicates used in each experiment has been mentioned in material and methods of the respective chapters.

2.8 Statistical analysis

The data were analyzed using ANOVA techniques and treatment means were compared (Chandel 1993).

2.9 Culture conditions

The cultures were incubated in culture room at 25±2 ^{0}C in dark or light (16 µE m^{-2} s^{-1}) and also in 24 h light condition at a light intensity of (27 µE m^{-2} s^{-1}). The incubation conditions have also been mentioned in each section separately.

2.10 Histological studies

Histological analysis was carried out by fixing the plant specimens (somatic embryos and immature embryo with shoot buds) in 5-10 ml (so as to dip the explant) of FAA (Formalin: acetic acid: 70% ethanol by volume) (5: 5: 90) in 15 ml capacity screw capped vials (Borosil®, India) for 48 hours at room temperature. Thereafter, the specimens were washed for 3-4 times with glass distilled water. Dehydration of the explants was carried out by passing them through t-butanol series (Sharma and Sharma 1980). This was followed by embedding in paraffin wax (melting point 58-60 ^{0}C) (Merck, E. Merck India Ltd., Bombay, India). Sections of 10 µm thickness were cut using a rotary microtome (Reichert-Jung 2050 Supercut, Germany) and specimens were fixed on slides by mild heating. The sections were then passed through the xylene - alcohol series (Sharma and Sharma 1980) and stained with 1% Heidenhein's hematoxylin (wt/vol. in distilled water, matured for one month in light) (Hi-Media Laboratories Pvt. Limited, Bombay) for one minute. The slides were counterstained with 1% eosin for two minute and mounted in DPX mountant. Histological sections mounted on slides were observed and photographed under a microscope (Docuval, Carl Zeiss, Germany).

Histological analysis procedure of the GUS stained sample was described in details in chapter 6.

2.11 Hardening of the plantlets

In vitro rooted shoots were carefully taken out from the agar medium of the test tubes and gently washed under tap water so as to remove the traces of agar and medium sticking to it. These rooted shoots were dipped in 1% aqueous solution of bavistin®, a systemic fungicide (BASF, India) for 10-15 min and then washed with tap water. Thereafter, the treated shoots were transferred in 8 cm earthen pots containing a mixture

of autoclaved soil and sand (1:1) or soil: sand: compost (1:1:1). The pots were covered with polypropylene bags and kept in green house. The plants were watered once in a week. The top corners of polypropylene bags were cut after two weeks to gradually expose the plants to the outside environment. After 3-4 weeks, the polypropylene bags were completely removed.

2.12 Scanning Electron microscopy

Samples were prefixed in 2% gluteral dehyde (Sigma) in 0.1 M cacodylate buffer (pH 7.2) (Sigma) for 48 h at room temperature, then washed with the same buffer 3 times and post fixed with 1% osmium tetroxide (OsO_4) (Sigma) in 0.1 M cacodylate buffer for 160 h at 4 ^{0}C. The samples were passed through distilled water, dehydrated in a graded acetone series (kept for 15 min each in 30%, 50%, 70%, 90% and 100% acetone). The samples were then placed on boats taking care not to allow drying by keeping them submerged in the transfer liquid (100% acetone) and loaded on the critical point dryer (Polaroid England), which was maintained at 20 ^{0}C. The chamber was filled with substitution fluid (liquid Co_2) and allowed to stand for 1 h for impregnation. The substitution fluid was allowed to evaporate by slowly heating the chamber to 36-38 ^{0}C. The Co_2 gas was carefully released and samples were removed and mounted on aluminum stubs using double sticky tapes (Bio-Rad, USA) or high conductivity paint (Acheson Colloids Company, England) sputter coated with gold-palladium (50-100 A^0) (Polar on coating unit E 5000, England). Scanning electron microscope was operated at an accelerating voltage of 10 or 20 kV.

2.13 Genetic Transformation and PRSV coat protein gene isolation studies

Details of materials and methods used for *Agrobacterium tumefaciens* mediated transformation and studies regarding isolation and cloning of PRSV gene have been described in the chapter 6.

CHAPTER 3

COMPARATIVE STUDIES ON *IN VIT*RO AND

IN VIVO SEED GERMINATION OF PAPAYA

3.1 Introduction

Micropropagation of plants *in vitro* aims at large scale production of true to type progeny. For this purpose explants from mature elite plants are preferred. However, the mature material is usually associated with heavy microbial contamination and exudation of phenolics that hampers the very induction of cultures. Moreover, mature explants are often recalcitrant and difficult to regenerate. Since the cells of mature explants are less plastic and fully differentiated, it is difficult to reprogram these and regain morphogenetic competence. It has been observed in several tree species that micropropagation from mature explants is not possible. For such species use of juvenile or rejuvenated material is necessary (Pierik 1987; Brar and Khush 1994; Ahuja 1994).

In vegetatively propagated plants, the culture of seed derived juvenile explants offers added advantage of introducing genetic combinations different from existing one. Continuos vegetative propagation of any plant results in loss of natural genetic variation and thereby narrowing the genetic base. This could create problems in crop improvement programs especially those involving quantitative trait loci (QTLs) such as high yield and disease resistance. New genetic combinations may have some useful traits that could prove valuable in crop improvement, as the availability of genetic variation is the prerequisite for any crop improvement program. *In vitro* seed germination is an important method in this regard as it facilitates establishment of aseptic culture explants for further culture.

Germination of seeds can be accomplished either by direct seed culture or by embryo culture.

Direct seed culture

Aseptic germination of seeds is an important method for obtaining juvenile materials of plants having seeds that are difficult to germinate (forest trees), having low or short viability (banana, plantains, black pepper); or require special mediators for seed germination (orchids, sandalwood).

Embryo culture

Embryo culture may be achieved either by culture of immature embryos or by culture of mature embryos. Immature embryos are mainly cultured from unripe or hybrid

seeds which fail to germinate, whereas mature embryo culture is mainly done to avoid inhibition in seed germination.

3.1.1 Relevance of *in vitro* seed germination and culture establishment in Papaya

Carica papaya L. is susceptible to a wide range of pathogens (Conover 1964), the biggest threat being the Papaya Ringspot Virus. The prevalence of the disease is world wide. Conventionally, papaya is propagated by seeds. Poor germination is a common phenomenon in papaya and it could be due to absence of embryos in 20% of seeds (Furutani and Nagao 1986). Breeders are under continuous pressure to produce disease resistant cultivars. Breeding programs are complicated by limited and variable seed germination of papaya (Chacko and Singh 1966; Lange 1961). This problem becomes serious when seeds of hybrids are to be used where the seed yield is very low. To ensure seed survival and viability, a high germination percentage of these seeds are necessary. In other way, the productivity of the crop can be increased by growing elite cultivars produced by *in vitro* clonal propagation. Need for *in vitro* explants arose because the establishment of cultures from explants grown outdoors is difficult due to severe contamination problems. A major constraint to the establishment of shoot bud cultures of papaya has been high associated bacterial contamination (Litz and Conover 1962; Mondal *et al*. 1990). *Pseudomonas* species and *Bacillus* species are the most commonly occurring contaminants in papaya shoot tip culture (Sharma *et al*. 1999). To avoid contamination, *in vitro* grown tissues are preferred over field grown materials (Mondal *et al*. 1994). Aseptic plant materials can be obtained *via* somatic embryogenesis, or direct germination of seeds and embryos. Although somatic embryogenesis has been reported in papaya (Fitch and Mansherdt 1990; Monmerson *et al*. 1995; Cheng *et al*. 1987; Cabrera-Ponce *et al*. 1996), the whole process of developing a new plantlet takes almost 2-3 month, whereas germination of seeds or embryos takes mere 7-10 days (Bhattacharya and Khuspe 2001). The process of seed germination could be exploited to preserve intrinsic genetic variability and to induce new combination by mutagenesis (Ravindran *et al*. 1985). It could also prove useful in obtaining contamination free source plants and juvenile explants that have better regenerability in tissue cultures. This aspects are vital for *Carica papaya* L. where cloning of adult, true to type field grown papaya plants is

difficult due to occurrence of large scale bacterial contaminations at stage I or stage II of micropropagation (Sharma *et al.* 1999).

In this chapter, we discuss *in vitro* and *in vivo* seed germination and the establishment of aseptic cultures of papaya.

3.2 Materials and Methods

3.2.1 Material used

Seeds of ten cultivars of papaya *viz*. Honey Dew, Pusa Majesty, Hybrid-781, Hybrid 786, Washington, Pusa Delicious, Disco, Pinky Flesh, Ex-15 and Pant Papaya, were collected from the Regional Fruit Research Station, Pune, India. Seed germination was studied in both *in vivo* and *in vitro* condition.

3.2.2 *In vivo* seed germination

In this study, different pre-sowing treatments and variation in temperatures were evaluated on *in vivo* germination of seeds**.**

3.2.2.1 Pre-sowing treatment

Prior to use, the pre-sowing treatments of the papaya seeds were soaking in water for 24 and 48 hour, acid scarification with 0.1N HCl (1 and 2 min), 0.1N HNO_3 (2 and 5 min), concentrated sulphuric acid H_2SO_4 (30 and 60 sec), soaking in gibberellic acid (GA_3) (100 and 200 ppm), naphthalene acetic acid (NAA) (100 and 200 ppm) and 0.1% potassium nitrate (KNO_3). Treatment of GA_3, NAA and KNO_3 was given for 24 hs. All experiments were carried out at room temperature. The seeds were not surface sterilized before using.

The seeds were washed with tap water to remove traces of any chemicals and were sown directly in soil filled tray at a depth of 3 cm. (Hereafter, direct sowing of seeds in soil will be referred as *in vivo* condition). Watering was done at five days intervals.

3.2.2.2 Temperature treatment

Germination of the seeds was tested at 15, 20, 25, 30, 35 and 40 ^{0}C. Seeds of all the varieties were sown in three replications. Each replication contained 100 seeds. Radical emergence was used as the criterion for germination. The observations on germination were recorded at weekly interval by taking out the seeds from the soil. Once germinated, the seedlings were removed and placed in the tray containing mixture of soil: sand: FYM (1:1:1).

3.2.3 *In vitro* seed germination

The effect of explant type, basal media, growth regulators, light conditions, gelling agents, support materials, antibiotics and genotypes on seed germination of papaya were studied in this chapter.

The seeds were kept under running water at room temperature for 1 h and then washed in liquid soap for 10 min. These seeds were then surface sterilized using ethyl alcohol (70%) for 30 secs followed by treatment with 4% sodium hypochlorite (NaOCl) for 10 second inside a laminar air flow cabinet. To remove traces of chlorine, the seeds were washed three times with sterile distilled water and then soaked in water for about an hour. These seeds were then placed on MS basal medium (Murashige and Skoog 1962) supplemented with 3% sucrose and a range of growth regulators. The medium was solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 by HCl or NaOH prior to autoclaving at 15 psi for 20 min at 121 ^{0}C. The medium was dispensed into 85 mm petridish. All the dishes were sealed with "Parafilm" and kept at 25±2 ^{0}C.

3.2.3.1 Effect of type of seed explant on papaya seed germination

Four different types of explants were used. These were intact and halved seed and intact and halved embryos. Seeds were cut longitudinally and the white embryo was taken out aseptically and cultured on MS basal medium to assess these for germination.

3.2.3.2 Effect of basal media on papaya seed germination

Seeds of ten papaya varieties were cultured on both agar solidified and liquid MS, Whites and B5 basal media to evaluate their effect on germination.

3.2.3.3 Effect of growth regulators on papaya seed germination

Effect of different concentrations of BAP, NAA, 2,4-D, 2,4,5-T, TDZ (0.1- 5.0 mg/l each) incorporated into the MS basal medium was evaluated on the germination of whole seeds and isolated embryos of papaya.

3.2.3.4 Effect of light conditions on papaya seed germination

Seeds and embryos of papaya were kept in both light (16 h photoperiod under cool white fluorescent tubes at 25 µmol m^{-2} s^{-1}) and dark conditions to assess the effect of light on germination.

3.2.3.5 Effect of gelling agents on papaya seed germination

Papaya seeds were kept in MS basal medium solidified with agar agar (0.75%) and phytagel (0.2% Sigma) and incubated in 16 h light and 8 h dark photoperiod condition.

3.2.3.6 Effect of support materials on papaya seed germination

Three different support materials i.e. filter paper, soil and cotton sterilized by autoclaving and soaked with MS basal medium, were placed in disposable sterile petridishes under sterile conditions and use for seed and embryo culture.

3.2.3.7 Effect of antibiotics on papaya seed germination

Effect of some commonly used antibiotics (cefotaxime, chloramphenicol, streptomycin and carbenicillin) was studied on seed germination of three papaya cultivars, Honey Dew, Pusa Delicious and Washington.

3.2.3.8 Effect of genotypes on papaya seed germination

The effect of genotype was studied by culturing all the ten varieties on MS medium in sterile disposable petridishes.

Germination event was marked by swelling of seeds or embryos, but seeds were designated to be germinated when radical or plumule emerged. The experiment was laid out with 3 replications in a randomised block design. Each plate contained 10 explants and each replication contained 30 explants. The progress of the germination was recorded for a period of 40 days at 7day intervals under a safe light condition. Statistical analysis of the data was done using ANOVA and treatment means were compared at 0.05 probability.

3.3 Results and Discussion

In this section, the result of *in vivo* seed germination have been described first, which is followed by the results of *in vitro* seed germination. The effect of each treatment has been described separately as written in the materials and methods sections.

3.3.1 *In vivo* seed germination

The seeds of all the varieties imbibed water and swelled within 5-6 days. When sown in soil, the commencement of seed germination (i.e. radical protrusion) was observed after 16-20 days, depending upon the papaya cultivar (Fig. 3.1). Emergence of radicals continued intermittently for about 40 days. Over that time 3 to 71% germination

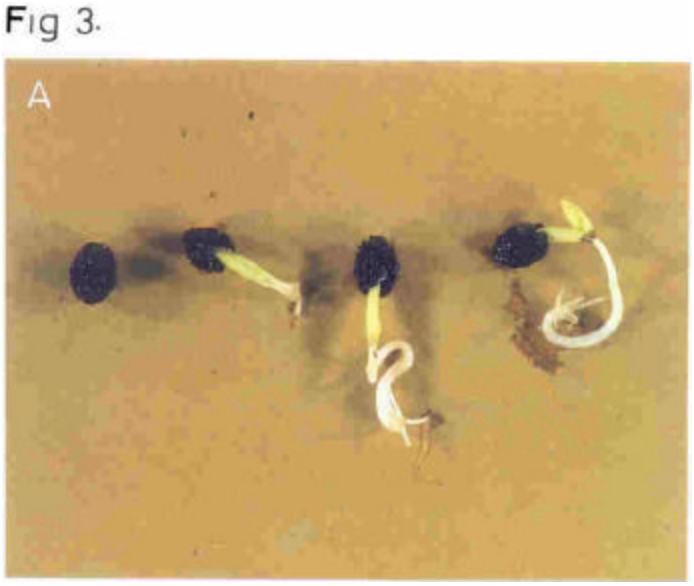


Fig.3.1

A. Stages of papaya seed germination cv. Washington.

(average 40.2%) was observed, depending upon the cultivar (Table 3.1). All the *in vivo* germinated seedlings developed into healthy plantlets.

Table 3.1 Start of germination and percentage germination after 40 days of *in vitro* and *in vivo* papaya seed germination.

Cultivar used	Days for commencement of germination		Mean	% germination after 40 days from sowing		Mean
	In vivo	*In vitro*		*In vivo*	*In vitro*	
Pusa Majesty	18.30 ± 1.50	5.30 ± 0.60	17.20	41.30 ± 4.70	74.00 ± 3.00	56.30
Pinky flesh	20.70 ± 4.00	6.00 ± 1.00	15.80	48.70 ± 3.50	74.00±4.60	61.30
Puss Delicious	19.30 ± 1.50	8.00 ± 1.00	18.50	44.00± 7.00	73.30 ± 4.50	51.80
Ex-15	17.70 ± 2.50	6.00 ± 1.00	18.00	16.30 ± 2.10	74.00 ± 3.60	42.70

Disco	16.70±1.50	9.30 ± 3.20	22.50	3.30± 4.20	71.30 ± 3.00	28.70
Hybrid 781	18.70 ± 1.10	8.70 ± 2.90	21.70	39.30 ± 4.20	71.70 ± 5.50	48.30
Hybrid 786	18.00± 1.00	6.00 ± 1.70	20.20	45.70 ± 1.10	73.30 ± 4.20	47.00
Honey Dew	16.70± 1.50	7.30 ± 0.60	18.80	70.70 ± 4.90	77.00 ± 1.70	69.80
Washington	17.70 ± 2.50	7.00 ± 0.00	18.80	49.00 ± 5.20	70.30 ± 0.60	52.00
Pant Papaya	19.70 ± 1.50	6.70 ± 3.00	22.00	43.00 ± 6.00	71.00 ± 3.00	54.0
Mean	18.33	7.03		40.16	59.48	

CD for genotype	4.5	CD for genotype	15.38
CD for conditions	35.5	CD for conditions	38.64
CD for genotype x condition	2.0	CD for genotype x condition	21.76
CD for treatment	5.8	CD for treatment	20.13

3.3.1.1 Effect of pre sowing treatment

Among all the presowing treatments given to enhance the papaya seed germination, soaking of seeds in 200 ppm GA_3 for 24 hs was found to be the best for all the cultivars except Ex-15, where the best result was obtained with GA_3 100 ppm (Table-3.2). Sen and Ghunti (1977) also reported that GA_3 was the best pre-sowing treatment to be given to non-chilled seeds. Positive effect of GA_3 for improving the papaya seed germination was also obtained earlier (Furutani and Nagao 1987; Tseng 1991). GA_3 is also found to affect positively the seed germination of other crops (Zarad *et al.*1997; Kuwahara *et al*. 1999). GA_3 generally acts through breaking of dormancy by inhibiting the action of ABA. It is possible that papaya seeds were dormant and therefore germinates in presence of GA_3.

Table 3.2 Percentages *in vivo* papaya seed germination after various pre- sowing treatments

Cultivar used	Control	Water soaking		HCl scarification		HNO_3 scarification		H_2SO_4 scarification		24 h GA3 dipping	
		24 h	48 h	1 min	2 min	2 min	5 min	30 sec	60 sec	100 ppm	200 ppm
Pusa Majesty	41.30 ± 4.70	45.00 ± 6.00	48.00 ± 2.00	17.30 ± 1.50	18.70 ± 1.10	60.00 ± 2.00	11.70 ± 2.90	7.00 ± 1.70	6.00 ± 2.60	50.00 ± 0.00	70.30 ± 4.00
Pinky flesh	48.70 ± 3.50	55.70 ± 4.00	58.00 ± 2.60	18.00 ± 4.60	14.30 ± 3.20	58.70 ± 2.10	11.30 ± 3.20	5.30 ± 0.60	3.30 ± 1.50	56.00 ± 3.60	61.30 ± 2.10

Pusa Delicious	44.00 ± 7.00	67.00 ± 1.00	47.70 ± 4.60	30.00 ± 3.00	53.00 ± 2.00	66.70 ± 3.20	16.70 ± 1.50	7.70 ± 2.50	6.30 ± 3.00	48.00 ± 3.60	67.30 ± 4.50
Ex-15	16.30 ± 2.10	17.70 ± 2.50	19.30 ± 8.10	2.70 ± 2.50	2.60 ± 2.10	9.70 ± 2.50	10.00 ± 2.00	4.30 ± 1.10	3.00 ± 1.00	21.70 ± 3.00	21.00 ± 5.30
Disco	3.30 ± 4.20	4.30 ± 4.90	5.00 ± 4.30	1.30 ± 1.50	2.00 ± 1.00	6.00 ± 2.60	9.30 ± 1.10	6.30 ± 1.10	3.00 ± 2.60	8.30 ± 2.80	12.30 ± 1.50
Hybrid 781	39.30 ± 4.20	42.70 ± 2.50	39.60 ± 6.60	31.70 ± 4.20	27.70 ± 2.10	38.70 ± 3.50	6.70 ± 1.50	4.70 ± 0.60	1.70 ± 1.50	45.00 ± 1.00	54.30 ± 5.80
Hybrid 786	45.70 ± 1.10	49.00 ± 6.00	51.00 ± 3.50	32.70 ± 2.00	51.00 ± 4.00	54.00 ± 4.60	11.70 ± 2.90	5.00 ± 0.00	4.00 ± 1.00	52.70 ± 2.50	62.70 ± 2.50
Honey Dew	70.70 ± 4.90	70.00 ± 0.00	73.30 ± 1.50	32.30 ± 4.00	36.70 ± 5.00	65.70 ± 5.10	10.00 ± 0.00	6.70 ± 2.90	3.00 ± 0.00	70.70 ± 2.50	79.00 ± 4.60
Washington	49.00 ± 5.20	51.70 ± 2.90	53.70 ± 6.00	36.30 ± 3.00	42.30 ± 5.00	56.70 ± 5.70	13.70 ± 1.50	9.30 ± 4.90	5.30 ± 4.20	56.70 ± 2.10	69.30 ± 4.00
Pant Papaya	43.00 ± 6.00	45.00 ± 5.00	47.00 ± 5.30	35.70 ± 4.20	34.70 ± 1.50	49.00 ± 3.60	11.70 ± 1.50	7.70 ± 2.50	2.30 ± 0.60	48.30 ± 5.80	67.30 ± 2.50

CD for Genotype = 5.3, Treatment=3.7,Time =2.3,Genotype treatment interaction, Genotype time interaction =Non significant, Treatment time interaction =5.3, Genotype,treatment and time interaction = Non significant.

The next best pre-sowing treatment in papaya was found to be acid scarification with 0.1N HNO_3 for 2 min (Table 3.2). Among the three acid tested, scarification with HNO_3 gave better result compared to control except Ex-15, Hybrid 781 and Honey Dew. General observation was that acid treated seeds germinated more uniformly than untreated ones (control). The use of acid treatment for enhancing seed germination was exemplified earlier (Purseglove 1974).

In our study, nitric acid treatment for 2 min was best while all other acid treatments gave less germination percentage. This may result from damage of the embryo because of acid treatment. The increase in germination percentage by acid treatment may be due to softening of hard testa which resulted in better water absorption and also helped in better gaseous exchange. Acid treatments also cause removal of inhibitory substances (Nagaveni and Srimathi 1980) which helps in germination of a seed. Use of nitric acid was also proved to be a superior acid treatment in seed germination studies of several crops (Naidu *et al.* 1999; Seeber and Agpaoa 1976). KNO_3 and NAA were both found to

be inhibitory to papaya seed germination. A similar negative effect with KNO_3 was observed in lime (Leonel and Rodrigues 1999) and with NAA in Phalaenopsis hybrid when used as pre germination treatment (Bhattacharjee *et al*. 1999).

3.3.1.2 Effect of temperature

Temperature was found to strongly influence the papaya seed germination. None of the cultivars germinated at a temperature of 15 ^{0}C and above 40 ^{0}C. Germination percentage was low at 20 ^{0}C and increased with increasing temperature to a maximum of about 80% at 30 ^{0}C. Above 30 ^{0}C, in all the varieties percentage germination decreased with temperature and was very low at 40 ^{0}C (Table 3.3). These findings confirm the findings of Yahiro and Yoshitaka (1982). For most of the cultivars, temperature of 25 ^{0}C was found to be more suitable than 35^{0}C for seed germination. However, this finding is in contrast with the findings of Furutani and Nagao (1987) who reported that heating up to 35 ^{0}C gave a higher percentage of seedling emergence of papaya seeds than heating up to 25 ^{0}C in *in vivo* condition. The percentage of any seed to germinate was maximum at the optimal temperature for that species. As the temperature declines or advances from the optimal temperature, two things may happen at the same time. While the percentage of seeds to germinate decreases, the number of days to germination increases. For every species of seed, there is an optimal soil temperature for germination, and at that temperature, the maximum number of seeds will germinate and in less time than at any other temperature.

Table 3.3 Effect of temperature (^{0}C) on papaya seed germination

Varieties	**20^0**	**25^0**	**30^0**	**35^0**	**40^0**
Pusa Majesty	25.00±1.00^{a}	52.33±0.58 ^{a}	81.00±1.00^{a}	28.00±1.00 ^{a}	4.66±0.58.^{b}
Pinky flesh	18.00±2.00^{b}	35.33±0.58 ^{c}	73.66±0.58^{def}	23.66±1.15^{cde}	0.33±0.58 ^{d}
Pusa Delicious	14.66±0.58^{d}	40.33±0.58.^{b}	76.33±1.53 ^{d}	18.33±0.50 ^{f}	8.00±1.00 ^{a}
Ex-15	24.33±1.15 ^{a}	33.00±1.00 ^{d}	71.33±0.58 ^{g}	27.66±0.58.^{b}	4.33±1.15.^{b}
Disco	8.33±0.58 ^{f}	23.66±0.58 ^{h}	80.00±1.00.^{b}	25.66±0.58 ^{c}	3.33±0.58.$^{b\,c}$
Hybrid 781	9.66±0.58 ^{f}	25.66±1.15 ^{g}	73.33±1.52 ^{def}	23.33±0.58^{cde}	4.33±0.58.^{b}
Hybrid 786	12.33±0.58 ^{e}	33.66±1.52 ^{d}	76.00±1.00 ^{d}	25.66±1.52$^{c\,d}$	5.33±1.15 .^{b}
Honey Dew	14.33±0.58 ^{d}	27.66±0.58 ^{f}	77.66±1.15 ^{c}	18.33±0.58 ^{f}	5.00±1.00.^{b}

Washington	17.66±0.58 [c]	22.00±0.00 [i]	74.66±1.15 [de]	24.66±0.58 [c]	3.33±0.58. [b]
Pant Papaya	19.33±0.58. [b]	28.00±1.00 [e]	77.00±1.00 [d]	21.66±1.15 [d]	4.00±0.00. [b]

Values with same letter (a-f) in a column are not statistically different at P = 0.05

This is the lowest temperature at which germination will occur for that crop, but the length of time for germination will be much longer than at optimum temperatures (Relf 1997).

3.3.2 *In vitro* seed germination

Germination of seeds kept in *in vitro* started within 5-9 days (Table 3.1). The maximum *in vitro* germination percentage (77%) was observed with Honey Dew cultivar, whereas cultivar Pant papaya responded least (71.0%) in *in vitro* germination (Table 3.1). This observation was recorded after 40 days.

3.3.2.1 Effect of explant type on seed germination

Of the four types of seed material (intact and halved seed and embryo separately) (Fig. 3.2), intact embryo yielded the highest germination percentages (Table-3.4). All these seedlings arose directly from the zygotic embryos. No somatic embryogenesis was observed. Intact embryos showed highest germination percentage may be because of the removal of the hard seed coat. Hard seed coat prevents seed germination by interference with water uptake, gaseous exchange. Also seed coat supplies inhibitors to the embryo or prevent the exhibit of the inhibitors (Bewley and Black 1994). This also may be the reason behind less germination percent of intact seed. It is observed that when halved seeds are used, germination percent was increased over the intact ones. It appears that physical action of cutting the seeds in half did not impede development rather improves it by removing a part of hard seed coat. Some germination inhibiting substances are present in the cotyledons whose affect is reduced when the seeds are halved (Bewley and Black 1994). The lowest percentage of seedlings was produced by halved embryo.

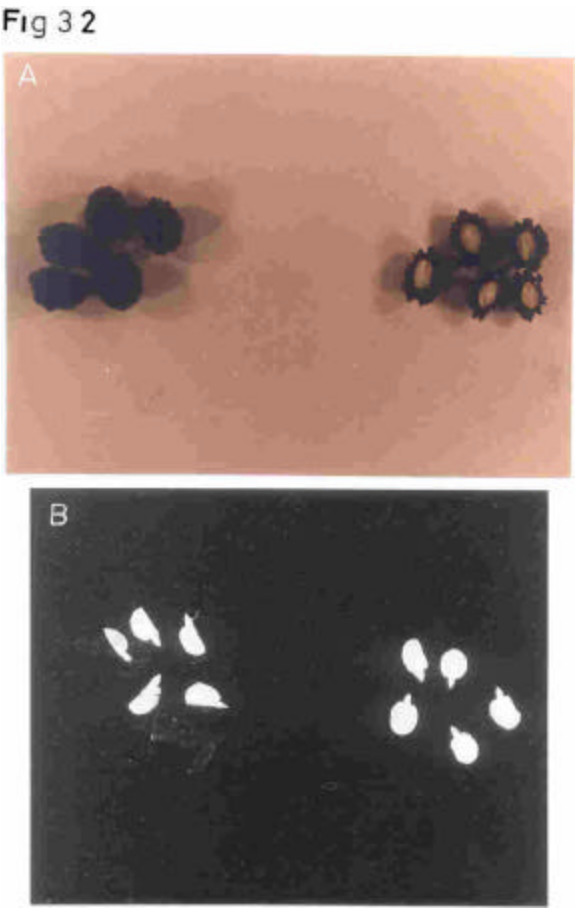


Fig.3.2

A. Intact and halved papaya seeds (cv. Washington)

B. Halved and whole embryos of papaya seeds (cv. Washington)

Table 3.4 Percentage *in vitro* germination of different papaya seed material in light and in darkness.

Cultivar used	% germination of intact seed		% germination of halved seed		% germination of naked embryo		% germination of halved embryo	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
Pusa Majesty	74.00 ± 3.00	70.70 ± 2.10	84.70± 2.50	81.30 ± 2.30	97.00 ± 2.60	95.30 ± 2.50	69.70 ± 5.00	65.00 ± 5.00
Pinky flesh	74.00 ± 4.60	73.70 ± 1.10	89.00 ± 5.60	86.00 ± 4.00	95.70 ± 3.00	91.70 ± 1.50	71.70 ± 5.80	66.70 ± 5.70
Pusa Delicious	73.30 ± 4.50	63.00 ± 4.00	77.30 ± 3.80	74.70± 2.50	97.70 ± 2.10	96.00 ± 1.00	67.33± 2.50	66.00 ± 3.60
Ex-15	74.00 ± 3.60	71.30 ± 3.20	83.70 ± 5.00	83.00 ± 3.60	94.00 ± 1.70	89.70 ± 3.50	73.70 ± 3.20	70.30 ± 2.50
Disco	71.30 ± 3.00	56.30 ± 4.00	72.00 ± 1.70	68.00 ± 3.00	92.00 ± 5.60	85.30 ± 3.50	65.00 ± 5.00	61.00 ± 3.60
Hybrid 781	71.70 ± 5.50	61.00 ± 5.30	70.00 ± 2.00	75.30 ± 3.50	95.30 ± 1.50	92.70 ± 2.50	62.30 ± 3.00	57.70 ± 2.50
Hybrid 786	73.30 ± 4.20	56.70 ± 4.70	65.30 ± 2.50	70.70± 4.10	96.30 ± 2.10	95.00 ± 1.00	63.70 ± 4.00	59.00 ± 5.30
Honey Dew	77.00 ± 1.70	64.30 ± 4.00	75.00 ± 0.00	75.70 ± 4.50	100.00 ± 0.00	99.00 ± 1.00	66.30 ± 3.20	61.70 ± 2.90
Washington	70.30 ± 0.60	55.70 ± 4.00	70.70 ± 3.80	65.00 ± 5.00	94.00 ± 5.30	91.30 ± 3.20	63.00 ± 2.00	54.30 ± 5.10
Pant Papaya	71.00 ± 3.00	60.00 ± 5.00	69.30 ± 6.00	67.30 ± 2.50	93.30 ± 2.90	90.00 ± 4.30	62.70 ± 2.50	53.70± 2.90

CD for Genotype = 6.6, Treatment = 4.2, Treatment and condition interaction =3.0, Genotype treatment interaction =13.3 Genotype condition interaction-Non significant, Treatment condition interaction =6.0,Genotype, treatment and condition interaction=Non significant.

This may be because, while dissecting, damage may be caused in the meristematic region of the embryos. Similar pattern of growth of halved embryos was also observed in *Rubus* seed germination (Ke *et al*. 1985).

3.3.2.2 Effect of basal media

Out of the three basal media formulation for seed germination of papaya MS medium was found to be the best followed by B5 and Whites in all the ten cultivars after 10 days of incubation (Table 3.5). Although a wide range of basal medium can be used in tissue culture studies 70% of the successful cases were either MS salts or its derivatives These media contain high levels of salts and both NH_4^+ and NO_3^-, the importance being recognized since 1965 (Thorpe 1988). Among the three media tested maximum germination was observed with MS medium, followed by B5 and White's media. The rate of root growth was higher in MS medium as compared to Whites or B5 medium.

Table 3.5 Effect of basal media on papaya seed germination

Varieties	MS	Whites	B5
Pusa Majesty	74.0 0± 3.00 [a]	47.00±2.00 [a]	57.00±2.00b [a]
Pinky flesh	74.00 ± 4.60 [a]	46.66±1.53 [a]	56.33±2.08 [a b]
Pusa Delicious	73.30 ± 4.50 [a]	46.33±3.78 [a]	59.66±2.08 [a]
Ex-15	74.00± 3.60 [a]	45.00±3.00 [a]	55.00±3.00 [ab]
Disco	71.30± 3.00 [a]	45.33±4.04 [a]	57.33±1.52 [a]
Hybrid 781	71.70± 5.50 [a]	47.00±0.00 [a]	57.33±0.58 [a]
Hybrid 786	73.30 ± 4.20 [a]	45.33±3.51 [a]	58.66±3.51 [a]
Honey Dew	77.00 ± 1.70 [a]	45.66±4.50 [a]	54.66±4.16[ab]
Washington	70.30 ± 0.60 [a b]	44.00±1.00 [a]	62.33±3.78 [a]
Pant Papaya	71.00 ± 3.00 [a]	44.66±2.08 [a]	55.00±2.00 [ab]

Values with same letter (a-b) in a column are not statistically different at P=0.05

Effectiveness of MS basal medium was also reported earlier in other crops (Chengalrayan 1997; Banerjee 2001).

3.3.2.3 Effect of growth regulator

Germination of the seed is known to be prevented by naturally occurring abscisic acid (ABA) blocking the stimulation of germination by gibberellin. It has been demonstrated that in some plants the exogenous application of cytokinin overcome the inhibitory action of ABA in seed germination (Khan *et al*. 1971). Among the hormones tested for *in vitro* germination of papaya seeds (BAP, NAA, TDZ, 2,4-D and 2,4,5-T) each at 0.1-5.0 mg/l, TDZ showed the best response (Fig. 3.3 A) in lowest concentration in all the cultivars. Highest germination percentage was obtained in EX-15 cultivar (95%) and lowest being Hybrid 786 (89.66%) (Table 3.6). When highest concentration of TDZ (5mg/l) was used, the embryos did not germinate at all. Granular white callus formed which covered the whole embryo gradually.

Table 3.6 Effect of TDZ on papaya seed germination

Varieties	Germination percent in presence of TDZ (mg/l)					
	0.1	0.2	0.5	1.0	2.0	4.0
Pusa Majesty	40.66±1.52 [a]	93.66±0.58 [b]	88.00±1.00 [ba]	74.66±0.58 [b]	64.33±1.52 [b]	14.66±0.58 [c]
Pinky flesh	41.00±1.00 [a]	92.00±1.00 [c]	90.00±1.00 [a]	73.00±1.00 [bc]	63.66±1.15 [b]	16.66±1.52 [b]
Pusa Delicious	40.33±1.15 [a]	97.33±0.58 [a]	87.66±0.58 [c]	74.00±1.00 [b]	63.66±1.52 [b]	9.00±1.00 [e]
Ex-15	40.66±1.52 [a]	95.00±1.00 [b]	90.00±1.00 [a]	81.00±1.00 [a]	66.00±1.00 [a]	11.33±0.58 [d]
Disco	39.00±1.00 [a]	93.33±1.53 [bc]	90.00±1.52 [a]	75.66±1.15 [b]	66.33±1.52 [a]	11.66±1.52 [d]
Hybrid 781	39.33±1.52 [a]	92.33±0.58 [c]	89.66±1.52 [b]	70.66±1.00 [e]	61.66±0.58 [c]	20.66±2.08 [a]
Hybrid 786	40.33±0.58 [a]	89.66±0.58 [d]	89.66±0.58 [ab]	72.00±0.58 [d]	65.33±1.15 [a]	08.33±1.15 [e]
Honey Dew	39.00±0.00 [a]	90.33±1.15 [d]	91.66±0.58 [a]	74.33±1.15 [b]	62.00±1.00 [bc]	07.33±0.58 [e]
Washington	40.00±1.00 [a]	90.66±0.58 [d]	90.33±0.58 [a]	73.66±1.15 [bc]	66.00±1.00 [a]	21.00±1.00 [ae]
Pant Papaya	39.66±2.08 [a]	90.33±0.58 [d]	87.00±1.00 [cd]	71.33±1.15 [d]	64.00±1.00 [b]	18.33±0.58 [b]

Values with same letter (a-e) in a column are not statistically different at P = 0.05

Table 3.7 Effect of NAA on papaya seed germination

Varieties	Germination percent in presence of NAA (mg/l)					
	0.1	0.2	0.5	1.0	2.0	4.0
Pusa Majesty	31.00±1.00 [a]	75.00±1.00[b]	77.00±1.00[b]	84.00±1.00[c]	56.00±1.00 [f]	35.66±1.15[c]
Pinky flesh	32.00±3.0[a]	72.00±1.00[d]	77.33±0.58[b]	81.66±1.58[e]	59.33±1.52[e]	47.00±1.00[b]
Pusa Delicious	30.66±3.05[a]	72.66±0.58[d]	69.33±1.52[d]	79.33±1.52 [f]	62.00±2.00[d]	52.33±0.58[a]
Ex-15	31.00±1.0[a]	75.66±2.08[b]	74.33±1.15[c]	85.66±0.58[b]	72.33±1.52[ab]	34.33±1.15[c]
Disco	30.66±2.51[a]	77.00±1.0[a]	80.66±0.58[a]	83.66±0.58[cd]	73.66±1.15[a]	29.33±0.58[cd]
Hybrid 781	32.00±.0.00[a]	78.66±1.15[a]	74.33±1.15[c]	90.33±1.52[a]	69.00±1.00[c]	19.00±1.00[e]
Hybrid 786	31.33±1.52[a]	76.33±0.58[b]	72.66±1.15[c]	86.00±1.00[b]	68.66±1.52[c]	17.66±1.15[ef]
Honey Dew	31.00±1.00[a]	71.33±1.52[d]	75.00±0.00[bc]	87.66±±1.15[b]	74.66±1.52[a]	18.00±1.73[e]
Washington	32.00±1.00[a]	74.66±1.15[bc]	74.66±1.15[c]	84.33±1.15[c]	72.00±1.00[b]	32.33±1.52[c]
Pant Papaya	32.00±1.73[a]	75.33±0.58[b]	76.00±1.73[b]	86.66±1.52[b]	74.66±0.58[a]	31.00±1.73[c]

Values with same letter (a-f) in a column are not statistically different at P = 0.05

Table 3.8 Effect of BAP on papaya seed germination

Varieties	Germination percent in presence of BAP (mg/l)					
	0.1	0.2	0.5	1.0	2.0	4.0
Pusa Majesty	33.66±1.52 ^{ab}	87.66±0.58^{a}	77.66±0.58^{a}	71.33±1.52^{b}	42.66±2.08^{e}	28.00±1.00^{a}
Pinky flesh	38.00±1.73^{a}	82.00±0.00^{cd}	73.33±0.58^{bc}	62.00±1.00^{d}	51.66±1.52^{bc}	29.00±1.00^{a}
Pusa Delicious	36.00±1.73^{a}	86.33±1.52^{a}	75.00±1.00^{b}	68.00±1.00^{c}	53.66±2.08^{b}	16.33±1.15^{de}
Ex-15	37.33±1.52^{a}	84.33±1.52^{b}	74.66±1.15^{b}	73.00±1.00^{b}	46.33±1.52^{d}	23.66±1.52^{b}
Disco	35.33±3.00^{a}	86.66±0.58^{a}	75.33±1.15^{b}	75.66±0.58^{a}	53.33±1.52^{b}	17.33±0.58^{d}
Hybrid 781	36.33±2.08^{a}	83.00±1.73^{c}	74.0±0.00^{b}	73.00±1.00^{b}	54.33±0.58^{ab}	18.33±1.15^{d}
Hybrid 786	37.66±2.30^{a}	87.66±1.15^{a}	74.66±1.52^{b}	70.66±0.58^{b}	55.00±1.00	24.66±0.58^{b}
Honey Dew	36.33±0.58^{a}	84.00±1.73^{b}	74.66±1.52^{b}	68.00±2.00 ^{c}	46.66±1.52^{d}	22.33±0.58^{bc}
Washington	37.33±2.08^{a}	85.66±1.52^{b}	72.33±1.00^{c}	74.33±1.52^{ab}	45.00±2.00^{d}	21.33±1.52^{c}
Pant Papaya	37.00±1.0^{a}	82.66±0.58^{c}	78.0±1.00^{a}	63.66±1.52^{d}	57.00±2.64^{a}	18.33±0.58^{d}

Values with same letter (a-e) in a column are not statistically different at P = 0.05

This was followed by NAA (Table 3.7) and BAP (Table 3.8) supplemented in MS basal medium. Hormones play an important role in seed germination process (Chee 1994). BAP at lowest concentration showed the maximum germination percentage whereas with NAA 5 mg/l gave the highest germination in all cultivars. Treatment with the higher concentrations of BAP and NAA and all concentrations of 2,4-D and 2,4,5-T in our study favored callus growth. Auxins in general were found to show a negative effect on seed germination of several crops (Bhattacharjee *et al*. 1999) while BAP is reported to help in germination by acting as an inhibitor of ABA (Bewley and Black 1994). The presence of TDZ in the medium was found to have a positive effect on germination of various crops (Murthy and Saxena 1998). The highest germination percentage was observed when low TDZ concentration (0.2 mg/l) was used. Not all the germinated seeds developed into whole plants. Some (7-8%) produced roots with stunted shoots when high concentration of TDZ (4.0mg/l) was used. A similar trend was also

observed by Massimo *et al*. (1995) in geranium, where an increase in concentration of TDZ increased the number of stunted seedlings.

3.3.2.4 Effect of light conditions

In our study, seeds germinated faster in the 16 h photoperiod than in darkness, thus confirming the importance of light in papaya seed germination. In cotton, Tort (1996) concluded that illumination significantly increased the germination percentage. Compared to darkened conditions 16 h photoperiod was found to increase germination percent (Table 3.4) and the root growth. Positive effect of light governing seed germination was observed earlier in other crops (Hand *et al*. 1992; Van der Sman *et al*. 1992). The importance of light as a factor in seed germination has long been recognized. Light sensitivity of seed was suggested as having some relations to seed germination in their natural habitat (Mayer and Polijakoff- Mayber 1979).

3.3.2.5 Effect of gelling agent

Use of different gelling agents used to solidify MS medium affected the germination percentage. Agar (0.75%) was found to be better suited than phytagel (0.2%) as a gelling agent for germination medium since it supported higher germination percentage (Table 3.9) (Fig. 3.3B) in all the varieties tested.

Table 3.9 Effect of gelling agent on papaya seed germination

Varieties	Gelling agent	
	Agar	Phytagel
Pusa Majesty	74.00 ± 3.00 [a]	57.66 ± 1.52[a]
Pinky flesh	74.00 ± 4.60[a]	56.00 ± 2.00 [a]
Pusa Delicious	73.30 ± 4.50[a]	57.33 ± 4.16[a]
Ex-15	74.00 ± 3.60[a]	57.00 ± 1.00[a]
Disco	71.30 ± 3.00[a]	55.66 ± 1.52[a]
Hybrid 781	71.70 ± 5.50[a]	55.33 ± 1.52[a]
Hybrid 786	73.30 ± 4.20[a]	58.33 ± 0.58[a]
Honey Dew	77.00 ± 1.70[a]	54.66 ± 2.30 [ab]
Washington	70.30 ± 0.60 [b]	53.66 ± 0.58 [b]
Pant Papaya	71.00 ± 3.00[a]	55.66 ± 0.58[a]

Values with same letter (a-b) in a column are not statistically at P = 0.05

Different gelling agent acts differently. The gellan type of gelling agents such as Gelrite and Phytagel (Sigma) are known to induce hyperhydricity (Podwyszynska and Olszeweski 1995). Though responsible for higher water availability and hyperhydricity in tissue cultures, phytagel was found to be inferior to agar as gelling agent in seed germination. The positive effect of agar on seed germination may be due to impurities in agar that acted as medium supplements (Podwysznska and Olszeweski 1995).

3.3.2.6 Effect of support material

Different support materials used for seed germination affected the germination percent considerably. Soil which is generally used to support seed germination *ex vitro*, showed lowest germination percentage in our *in vitro* seed germination experiment. Use of cotton and filter paper gave satisfactory germination percentage. Filter paper was found to be the best support material for papaya seed germination (Table 3.10) (Fig. 3.4 A).

Fig. 3.3

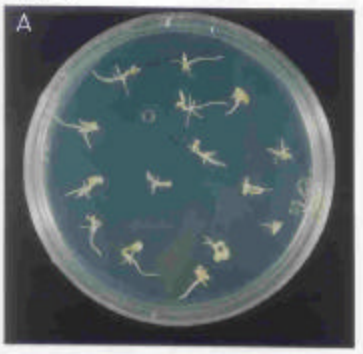


Fig. 3.3

A. Germination of papaya embryos in presence of TDZ in the MS basal medium

B. Germination of papaya embryos in presence of agar as gelling agent in MS basal medium

Table 3.10 Effect of substrate on papaya seed germination

Varieties	Substrate		
	Soil	Filter paper	Cotton
Pusa Majesty	29.66±2.30[a]	79.00±3.00 [a]	53.00±4.35[a]
Pinky flesh	30.66±2.30 [a]	75.66±1.15 [b]	53.66±4.72 [a]
Pusa Delicious	32.33±1.15 [a]	77.66±2.51 [a]	53.33±3.21 [a]
Ex-15	10.33±3.21 [b]	80.00±1.73 [a]	53.66±3.78 [a]
Disco	0.33±0.58 [c]	78.33±3.51 [a]	54.66±3.78 [a]
Hybrid 781	33.33±2.30 [a]	78.00±2.00 [a]	53.66±3.78 [a]
Hybrid 786	33.33±2.08 [a]	80.00±3.46 [a]	53.00±3.46 [a]
Honey Dew	31.00±2.00 [a]	79.66±2.08 [a]	55.66±2.88 [a]
Washington	31.33±0.58 [a]	80.33±4.50 [a]	55.00±3.00 [a]
Pant Papaya	31.66±3.51 [a]	81.33±4.50 [a]	54.00±2.64 [a]

Values with same letter (a-b) in a column are not statistically different at P = 0.05

However, soil supported the best root growth followed by filter paper and cotton. Filter paper was effective in papaya seed germination may be because it could provide appropriate moisture necessary for the seeds to germinate as observed in seed germination of other crops (Prasad *et al*. 1996). Cotton holds excessive moisture, which may result in fungal contamination. Proper humidity can be maintained with filter paper as media can be replenished as and when needed. Sterilized soil used for *in vitro* germination was found to be less suitable than non autoclaved soil used in ex vitro germination. This suggests the positive effect of different biological and physico-chemical factors in non sterile soil used *ex vitro*. However, sterilized soil supported root growth better than cotton or filter paper.

3.3.2.7 Effect of antibiotics

Antibiotics also affect the papaya seed germination (Fig. 3.4 B). Four antibiotics were tested cefotaxime (100-1000 mg/l), carbenicillin (100-1000 mg/l), cholramphenicol (1-40mg/l), and streptomycin (100-1000 mg/l) on three cultivars, Honey Dew, Pusa

Delicious and Washington (Table 3.11). Among them, cefotaxime and carbenicillin were found to affect the germination in a positive way.

Table 3.11 Effect of antibiotics on papaya seed germination

Antibiotic	Concentration (mg/l)	Honey Dew % germination	Washington % germination	Pusa Delicious % germination
Cefotaxime	100	78.66±0.58^{c}	72.00±2.00^{b}	74.66±2.51^{b}
	250	82.33±1.53^{b}	86.00±1.00^{a}	76.66±2.08^{b}
	500	88.00± 1.00^{a}	58.00±1.00^{c}	85.66 ± 1.15^{a}
	750	61.00±1.00^{d}	47.00±2.00^{d}	46.33±1.52^{c}
	1000	42.66±3.05^{e}	32.33±2.51^{e}	27.00±1.00^{d}
Carbenicillin	100	79.33±1.52^{b}	70.33±2.08^{c}	70.66±1.52^{b}
	250	83.66 ±2.30^{a}	74.33±2.08^{b}	80.00±1.00^{a}
	500	84.00±1.00^{a}	81.33±2.08^{a}	80.66±1.52^{a}
	750	44.00±2.64^{c}	54.33±4.04^{d}	47.00±1.00^{c}
	1000	23.00±2.00^{d}	32.33±2.51^{e}	26.33±1.52^{d}
Control		77.00±1.70^{c}	70.30±0.60 ^{b}	73.30±4.50 ^{b}

Values with same letter (a-e) in a column are not statistically different at P = 0.05
CD value for Cefotaxime on Honeydew is 2.86, Washington-3.24 and on Pusa Delicious is 3.14. Again for Carbenicillin on Honey Dew is 3.56, Washington 4.79 and on Pusa Delicious 2.66

Maximum germination percent in the two cultivars Honey Dew (88%) and Pusa Delicious (85.66%) was observed at 500 mg/l cefotaxime whereas in Washington highest percent germination was achieved with cefotaxime 250mg/l. Higher concentration decreased the germination percentage. Carbenicillin at 500 mg/l showed increased germination percentage of Honey Dew (84%) and Washington (81.33%) and Pusa Delicious (80.66%). Above 500mg/l a negative effect was obtained either by inhibiting the germination or by abnormal germination.

Earlier in another study, carbenicillin (375-500mg/l) was found to have an inhibitory effect on somatic embryogenesis but promoted callus growth in papaya cultures (Yu *et al*. 2001). Also, at higher concentration carbenicillin was found to reduce shoot growth in other crops (Agrawal *et al*. 1998). The use of other two antibiotics, streptomycin and chloramphenicol was found to be completely inhibitory to papaya seed germination. In both the cases only initial swelling of the seeds were there but later on no germination was observed. Similar inhibitory action of streptomycin and chloramphenicol was also observed in other crops earlier (Bastian *et al*. 1983; Pollock *et*

al. 1983). The stimulatory effect of cefotaxime was also observed earlier in papaya (Yu *et al.* 2001) and other crops (Prediery *et al.* 1989). Growth regulatory activity of cefotaxime may be due to active molecule of cefotaxime that mimics a growth regulator (Mathias and Mukasa 1987) or due to the degradation of cefotaxime by plant estarases which might generate metabolites which account for growth regulator like activity (Mathias and Byod 1986). Similar to our study, both cefotaxime and carbenicillin belonging to *b*-lactam group have reported to cause minimal toxicity on most plant tissues (Mathias and Boyd 1986).

3.3.2.8 Effect of genotype

Besides environmental factors and its correlative effects the dormancy of a seed also depends on genetic control (Bewley and Black 1994). Germination behavior of papaya seeds was found to be genotype dependent. The lowest germinability in *in vivo* condition was observed in the cultivars Disco (3.3%) and highest in Honey Dew (70.7%). However, in *in vitro* condition lowest germination percent was observed in Pant papaya (71.0%) and highest in Honey Dew (77%) (Table 3.1). The rate of root elongation was found to be similar in almost all the cultivars. The cultivar specific germination observed in papaya could be due to genetic or environmental factors. Difference in germination may be due to underdeveloped embryo or absence of embryos characteristic to papaya (Furutani and Nagao1986). Similarly the cultivars may require optimum germination conditions and the conditions used in present study could be sub optimal for some of them.

Seed germination is considered to begin with imbibition of water by seed and end with elongation of embryo axis, usually the radical and rarely plumule (Bewley and Black 1994). According to this definition 90% of the seeds of papaya showed ability to germinate because they imbibed water. But about 20% of seeds, which have imbibed water, could not complete the process of germination because radical emergence was not observed from them. The embryos from such seeds could not elongate into seedlings but became brownish in colour and died. This may be due to presence of some germination inhibitors in papaya seeds (Nagao and Furutani 1986). Presence of embryoless papaya seeds has been reported in papaya earlier (Furutani and Nagao 1986). Besides environmental factors and its correlative effects the dormancy of a seed also depends on

genetic control (Bewley and Black 1994). Some post germination anomalies were observed in this germination study (Fig.3.5A). Root less seedlings (4%), unequal cotyledonary leaves (9%) and production of 2-3 seedlings from a single seed were observed but those abnormalities did not affect the growth of seedlings (Fig. 3.5 B and C). The normal seedlings produced by seeds under different treatments appeared similar

Fig. 3.4

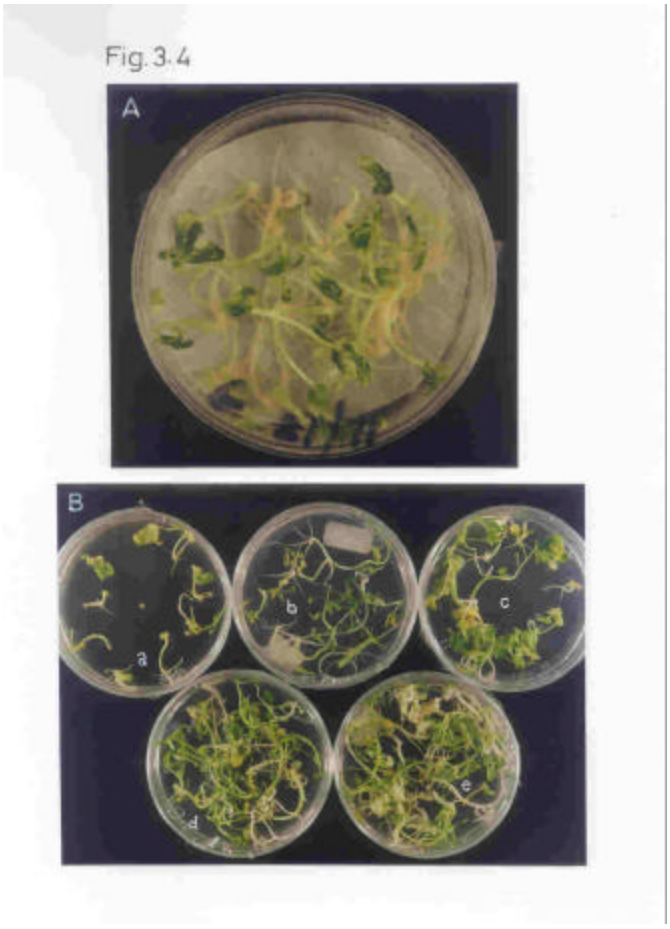
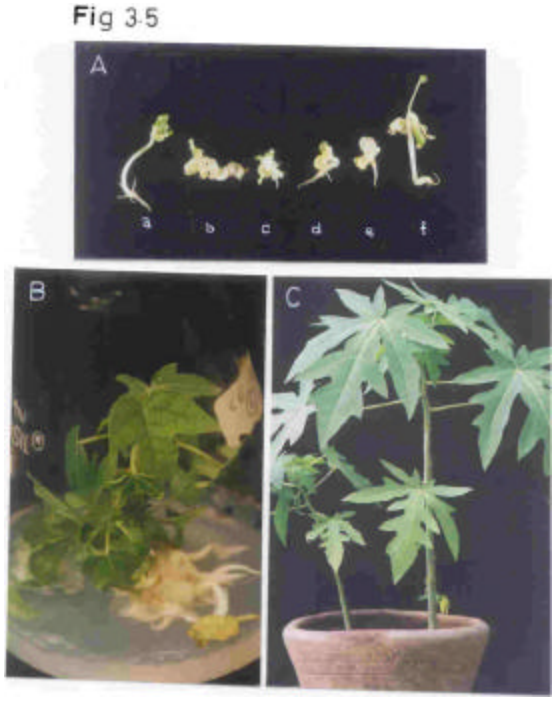


Fig. 3.4

A. Papaya seed germination with filter paper as support material

B. Papaya seed germination (cv. Honey Dew) as affected by presence of antibiotic in the germination medium, a-carbenicillin (1000 mg/l), b- control, c- cefotaxime (1000 mg/l), d- cefotaxime (500mg/l), e- carbenicillin (750mg/l)

Fig. 3.5



A. Abnormalities observed during papaya embryo germination, a-normally germinated seedling, b to f-abnormal seedlings

B. Normal papaya seedling (cv. Honey Dew) obtained from abnormally germinated embryos

C. Normal growth of papaya cv. Co-2 obtained from abnormally germinated embryos

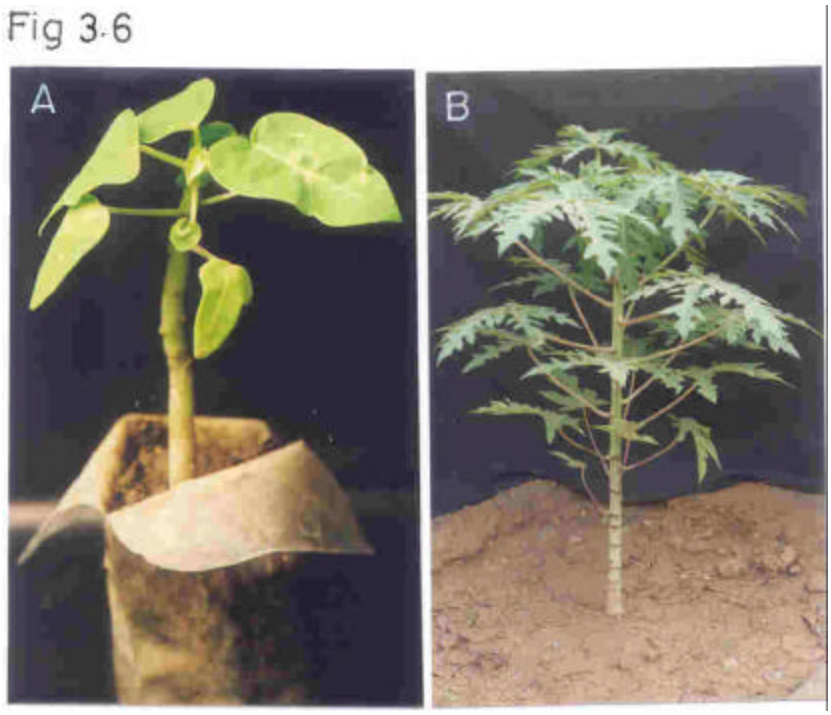


Fig.3.6

A. *Ex vitro* grown papaya seedling of cv Co-2.

B. *In vitro* germinated seedling of papaya cv. Washington after transplanting in field

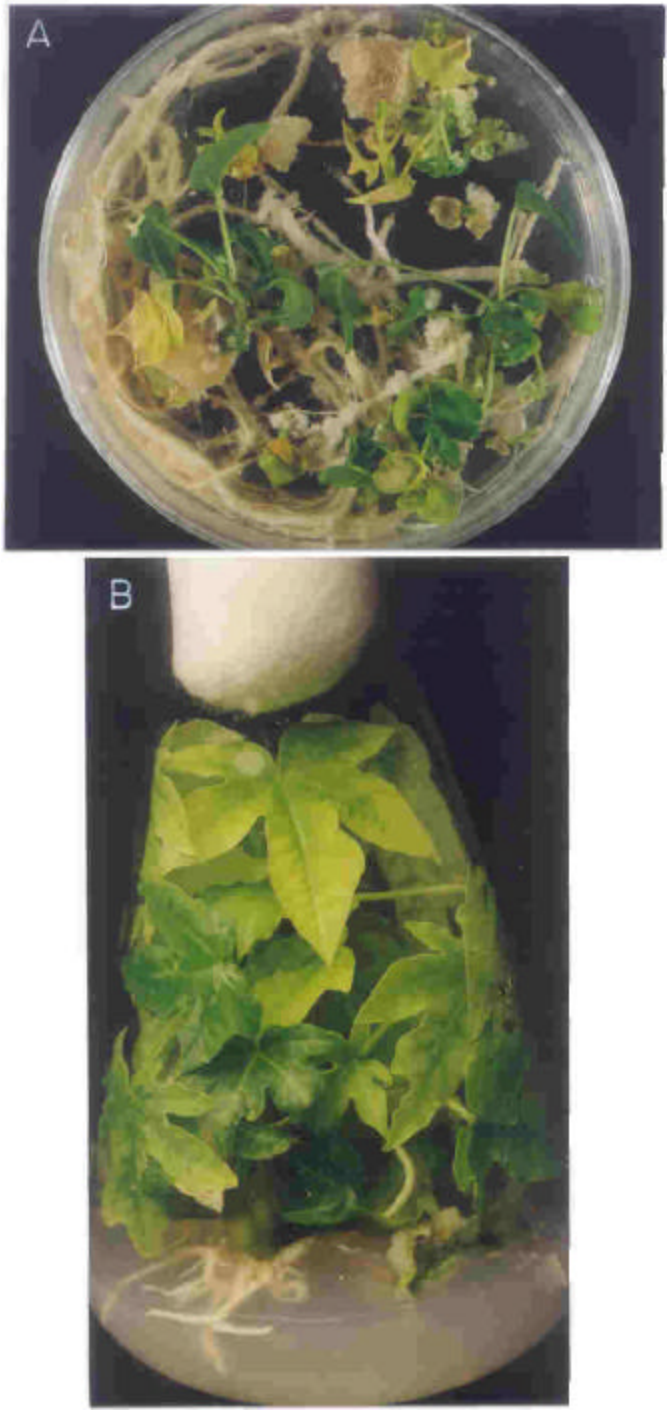


Fig.3.7

A. Three month old papaya seedling cv. Pusa Delicious growing in 85 mm petridish

B. Six month old papaya seedling cv. Co-2 in 250ml conical flask

3.4.Conclusion

Results presented here for *in vitro* conditions were found to quicken the process of seed germination over in-*vivo* conditions by about one week. Not only that, *in vitro* seed germination was more uniform compared to *in vivo* germination process. Also, no phenotypical difference was observed in *in vitro* and *ex vitro* grown seedlings (Fig 3.6). Germination of papaya seeds depends considerably on the pre treatment of seeds and the temperature at which the seeds are kept for germination. Effect of hormones, basal media, gelling agent, culture conditions, substrate, antibiotics and genotypes etc were also found to influence the *in vitro* rate of germination of papaya seeds. Present study reveals that TDZ can be suitably used to enhance the papaya seed germination rate in vitro. Thus the parameters optimized here would help in achieving successful germination and thereby production of sterile seedlings, which could be used in tissue culture for micropropagation and transformation studies. The seedlings also find application in preservation of inherent genetic diversity and also in production of novel genetic combinations by distant hybridization with other cultivated and wild species through protoplast technology or mutation breeding especially when a small amount of seed is available.

Part of the work is published as a paper entitled "*In vivo* and *in vitro* germination of papaya seeds" in Scientia Horticulturae (2001). 91(1-2): 39-49.

CHAPTER 4

INDUCTION OF SOMATIC EMBRYOGENESIS AND PLANT REGENERATION IN PAPAYA

4.1 Introduction

Somatic embryogenesis is a process through which embryos develop from somatic cells, without the fusion of gametes and later differentiates into plants through characteristic developmental pattern, a phenomenon, which is not observed in zygotic embryogenesis (Tisserat et al. 1979; Williams and Maheshwaran 1986; Rangaswamy 1986; Zimmerman 1993; Merkle et al. 1995). It is a pathway of de novo regeneration from in vitro cultured tissues either from callus (indirect somatic embryogenesis) or from cells of an organized structure such as stem, leaf, hypocotyl or zygotic embryo (direct somatic embryogenesis) (Williams and Maheshwaran 1986). The somatic cells within a plant contain the genetic information necessary to form a complete and functional plant (Merkle et al. 1995). The somatic embryos are bipolar structures, lacking a vascular connection with the mother tissue and resemble the zygotic embryo in giving rise to a new plant (Raghavan 1976). They consist of complete propagules (contains both the root and shoot pole requiring only a single step for the formation of an entire plant (Stuart and Redenbaugh 1987; Parrott et al. 1995). The initiation of somatic embryogenesis occurs with the termination of the existing gene expression pattern in the explant tissue and its replacement with an embryogenic gene expression program in those cells of the explant, from which somatic embryos arises (Merkle et al. 1995). Embryogenic cells which form after many divisions in culture and require growth regulators not only for their entry in mitosis again but also for the determination of the embryogenic state which are termed as IEDC's (induced embryogenic determined cells). Formation of IEDC's happen in case of indirect embryogenesis (Sharp et al. 1980; William's and Maheshwaran 1986). Conversely, direct embryogenesis in culture, proceeds from cells which are pre determined for embryogenic development, i.e. they are PEDC's (pre embryogenic determined cells). They require an external stimulus, either in the form of growth regulators or favourable conditions for the induction and development of embryos (Sharp et al.1980; William's and Maheshwaran 1986; Carman 1990). The formation of somatic embryos is dependent on the epigenetic state of the explant (Merkle et al. 1990; Litz and Gray 1995) as embryogenic and juvenile tissues of plants can be easily coaxed to form embryos in comparison to differentiated vegetative cells (Thorpe 1994).

The expression of somatic embryogenesis depends on a number of factors. Developmental stage, type of tissue explant and particular cell types affects the somatic embryogenesis. After the induction of embryogenic determined cells, there is no difference between direct and indirect somatic embryogenesis (Williams and Maheshwaran 1986). The embryogenic cells closely resemble the rapidly dividing meristematic cells of apical meristems, i.e. they are small celled and having a large nuclei with prominent nucleoli (Tisserat et al. 1979; Williams and Maheshwaran 1986).

The question of single or multiple cell origin of somatic embryos is directly related to the coordinated behavior of the neighboring cells as morphogenic groups (Williams and Maheshwaran 1986). Determined cells may operate singly or in groups to form embryos. In case of indirect somatic embryogenesis, either through callus or suspension culture, the origin of the embryos was found to from a clump of embryogenic cells called the proembryonel mass (PEM), from which embroids develop (Williams and Maheshwaran 1986). Direct somatic embryogenesis may also be multicellular (Haccius 1978) or may arise from a single superficial cell (Konar and Nataraja 1965) or by both of these pathways (Williams and Maheshwaran 1986). The direct somatic embryogenesis is advantageous over indirect pathway because embryos produced via callus phase may not produce true to type plants. However, useful variants could be isolated through indirect embryogenesis due to somaclonal variation (Rangaswamy 1986).

Auxin and auxin like substitutes are mainly used for the in vitro induction of somatic embryogenesis in various crops (Litz and Gray 1995). Although Picloram, Dicamba, NAA, 2,4,5-T are being used to induce embryogenesis, it is 2,4-D which is the most commonly used auxin that has been exploited for various crops (Fitch 1990, Durham and Parrott 1992). Cytokinin induced somatic embryogenesis is rare but has also been achieved (Maheshwaran and Williams 1986; Gill and Saxena 1992). Recently, TDZ has also been found to induce somatic embryogenesis (Murty et al. 1996). Remarkable progress has been made in the commercialization of somatic embryogenesis since its discovery in carrot by Steward et al. 1958. Following are some of the important uses and application of somatic embryogenesis in plant tissue culture.

1) Formulation of plant cells in fewer steps
2) Potensial for production of large number of plant.

3) Morphological and cytological uniformity of the plantlets (Vasil and Vasil 1986) and the promise of synthetic seed technology (Ammirato and Styer 1985).
4) Helps in crop improvement like selections for salt tolerance and disease resistance has proved efficient in embryologically competent callus tissues of citrus (Litz et al. 1985).
5) Embryo rescue technique helps to rescue embryos that normally abort in wide crosses and as a means of obtaining plants from intergeneric hybrid embryos (McGranahan et al. 1988; Ozias-Akins et al. 1992)
6) As more number of regenerants can be obtained originating from few or single cells through somatic embryogenesis, it thereby increases the likelihood of achieving more transformed cells.
7) Can be used to synthesize desirable metabolites through cell or organ culture like lipid synthesis, has been reported in embryo cultures in a number of species including cocoa, jojoba, borage, rape seed, carrot (Jannick 1991; Weber et al. 1992) and peanut (Mhaske and Hazra 1994).
8) Somatic embryo derived plants are free of pathogens including viruses (Janick 1993) because of their absence of vascular connections between the nucleus and other maternal tissues.
9) Large scale production of somatic embryos of agronomic crop and their packaging in artificial seeds to reduce the dependence on unpredictable seed set and seed viability (Gupta and Durzan 1987).
10) Last but not the least, somatic embryogenesis has become an important tool for basic plant biological studies as it can be used for molecular and biochemical events that occur during induction and maturation period of somatic embryo development.

4.1.2 Somatic embryogenesis studies in papaya: Current status

The earliest report of successful somatic embryogenesis in papaya was by De Bruijne *et al*. (1974). They were successful in induction of somatic embryos from petiole sections cultured on Murashige and Skoog (1962) and White's (1963) media in a multistep protocol. They obtained somatic embryos but were not able to regenerate plants. Later, many other authors (Yie and Liaw (1977), Mehdi and Hogan (1979), Chen *et al*. (1987), Chen (1988a,b) Yamamoto and Tabata (1989)) have used *in vitro* grown

seedlings as their source of explant for somatic embryo induction. Green house grown seedlings were also used as source of explant for inducing somatic embryos (Jordan *et al*. 1982). Fitch and Mansherdt (1990) have followed the protocol published by Litz and Conover (1981b, 1982, 1983) and also of Mansherdt and Wenslaff (1989 a, b) and could produce somatic embryos for their transformation studies. Somatic embryos were also induced from hypocotyl tissues by Yang (1988) and also by Fitch (1993). Although different explants were found to produce somatic embryos, immature embryos still remains the most preferred explant (Cai *et al*. 1999; Bhattacharya *et al*. 2002). Reports on papaya somatic embryogenesis has been summarized in chapter 1.

Literatures on somatic embryogenesis of Indian cv. of papaya are rather meager. Majority of the work published on somatic embryogenesis of papaya is on cultivars, which are not commonly cultivated in India. Hence, the present investigation is carried out with the aim of developing an efficient papaya plant regeneration system *via* somatic embryogenesis from immature and mature explants of the most commonly cultivated varieties of India. The developed protocol could be useful for synthetic seed technology and also for genetic transformation studies in Indian cultivars of papaya.

4.2 Materials and Methods

4.2.1 Plant material

Explants (immature and mature embryo) (Fig. 4.1) from two different sources were tested for somatic embryogenesis induction. Sterile immature seeds of Honey Dew, Washington and Co-2 were used for excision of embryos after cutting the fruits. These embryos were used directly for somatic embryo induction or were germinated on MS basal medium supplemented with 2.0mg/l of GA_3 (parts of the germinated seedling were used as source of other explants for somatic embryo induction).

Mature seeds of Honey Dew and Co-2 were surface sterilized as described in chapter 2. The surface sterilized mature seeds were soaked in sterile distill water for 18 h in the dark at 25±2 ^{0}C and kept on gyratory shaker at 200 rpm.

4.2.2 Explant preparation

For isolation of embryo, seed coat was removed inside the laminar flow by making a longitudinal cut with the sterile surgical blade and the embryo was taken out aseptically from seeds.

Fig. 4.1

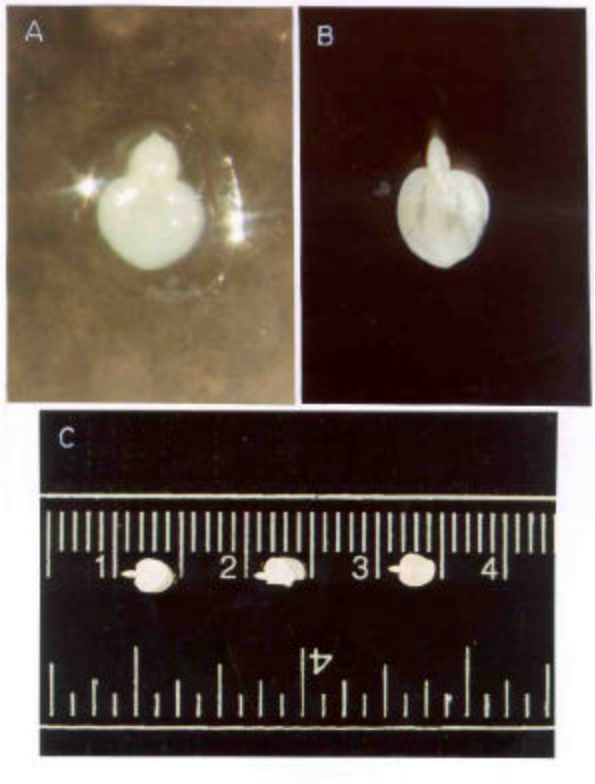


Fig. 4.1

A. Immature papaya embryo (90s day old) of cv. Honey dew.

B. Mature papaya embryo of cv. Honey dew

C. Normal size of the excised immature papaya embryo (110 days old) of cv. Honey dew

4.2.3 Induction of somatic embryogenesis

Induction of somatic embryogenesis has been tested in both immature and mature zygotic embryo explants.

4.2.3.1 From immature embryo

The effect of various auxins and cytokinins on induction of somatic embryogenesis from embryo axis of cultivars Honey Dew, Washington and Co-2 was evaluated. MS basal medium along with vitamins supplemented with 30g/l sucrose and various concentrations of growth regulators (2,4-D, 2,4,5-T, Picloram, Dicamba) was used as the embryogenesis induction medium. The pH of the medium was adjusted to 5.8 before autoclaving. The medium was solidified with 0.7% agar (HiMedia, India). Thirty ml medium was poured in each pre sterilized 85 mm diameter petridish and allowed to solidify. Immature zygotic embryos excised from the papaya seeds were cultured on the

induction medium. The cultures were incubated at 25±2 ^{0}C for 4 weeks with a 16 h photoperiod under cool white fluorescent light (25 µmol m^{-2} s^{-1}). The experiment was repeated thrice. Depending on the development of globular stage embryo response on various hormones, a number of parameters were further studied keeping the concentration of growth regulator and the cultivar as constant.

4.2.3.2 From mature embryo

Mature embryo of cultivars Honey Dew and Co-2 were tested for somatic embryo induction. In our earlier report (Bhattacharya *et al*. 2002), we have mentioned the induction of somatic embryogenesis from immature embryo axes. Since procuring immature explants may be difficult through out the year, mature embryos of the two cultivars were also evaluated for their somatic embryo induction potential. The explants were cultured on MS liquid media supplemented with 2,4-D, 2,4,5-T, Picloram and Dicamba. The pH of the medium was adjusted to 5.8 before autoclaving. Twenty ml medium was poured in each pre sterilized test tube with filter paper bridge. Two explants per test tube were inoculated. The cultures were incubated at 25±2 ^{0}C for 4 weeks under dark condition.

4.2.3.3 Effect of growth regulators on induction of somatic embryogenesis

In a preliminary experiment, different phytohormones were tested to assess the morphogenetic response of the explant. Immature and mature embryos were cultured in petridishes and test tubes with 30 ml and 20 ml medium respectively. Auxins like 2,4-D (1-25 mg/l), 2,4,5-T (1-20mg/l), Picloram (0.5-10mg/l), Dicamba (1-15mg/l) and combination of 2,4-D (1-5 mg/l) and 2,4,5-T (1 and 2 mg/l) were tested in immature embryos whereas 2,4-D, 2,4,5-T, Picloram and Dicamba (1-10 mg/l) were tested with mature embryos. In case of solid media, 30 explants and in case of liquid media, 20 explants (2 embryos/test tube) per treatment were studied and the experiment was repeated thrice. All the cultures were incubated as mentioned above for a period of 4 weeks. After 4 weeks, the nature of explants response in terms of globular embryos formed was recorded.

4.2.4 Development of cotyledonary structures and their maturation

Two hundred globular embryos were transferred for cotyledonary stage development in case of immature embryo axes explant and fifty globular embryos were

transferred in case of mature embryo axes. For further development and maturation, regenerated globular embryos were also transferred back to the same induction medium or in MS medium with 3% sucrose, 0.75% agar and ABA (0.1mg/l for 5 days).

4.2.5 Germination and conversion of the cotyledonary stage embryos

For germination, mature cotyledonary stage embryos were transferred to phytohormone free MS basal medium supplemented with 3% sucrose and 0.75% agar or into MS basal medium with 3% sucrose and 0.75% agar, supplemented with either absicic acid (0.1 mg/l) or GA_3 (1 mg/l) or Zeatin (1mg/l) or BAP (1.0 mg/l). The embryos were cultured at 25 ± 2^0 C under cool white fluorescent light at 25 mol. m^{-2} s^{-1} with 16 h photoperiod for 2-3 weeks for the development of root and shoot. The cultures were incubated under the conditions described as above for 2 weeks. Twenty embryos were cultured per treatment and the experiment was repeated thrice.

4.2.6 Developments of plantlets

Fully germinated embryos with well defined root and shoot were transferred to hormone free MS basal medium supplemented with 3% sucrose, 0.75% agar or 0.2% phytagel for elongation and development of whole plantlets. The cultures were incubated under the conditions described above for a period of 15 days.

4.2.7 Transfer of plantlets to soil

Plantlets (4-5 cm high) were taken out from the culture vessels, washed with de-ionized water gently and then is dipped in 0.1% (w/v) Bavistin (BASF India Limited, Mumbai, India), a broad-spectrum fungicide, for 1 min. The plantlets were then transferred to a pot (8 cm) with a potting mixture of sand, soil and farm yard manure (1:1:1 w/w). Plantlets were hardened under green house condition for 3-4 weeks and then planted out in field.

4.2.8 Statistical analysis

The data was analyzed using Analysis of Variance technique for completely randomized design and the treatment means were compared.

4.2.9 Histology and Scanning Electron Microscopy

For histological and SEM (scanning electron microscope) studies, the various developmental stages of somatic embryos were fixed and further studies were conducted as per the procedure described in chapter 2.

4.2.10 Parameter studies on immature embryo explant

Various parameters such as basal medium, explant source, carbohydrate source, light /dark incubation, gelling agents were tested for immature explants to optimize the conditions for induction of somatic embryogenesis of papaya. Each experiment is described separately.

4.2.10.1 Effect of different basal media

Five different basal medias such as MS, B5, ½ MS, Whites and MS basal media +B5 vitamin supplemented with 2 mg/l glycin (hereafter this will be referred to as MBG) with 3% sucrose, 0.75% agar were used to test the induction of somatic embryogenesis. The cultures were incubated at 25±2 ^{0}C for 4 weeks with a 16 h photoperiod under cool white fluorescent light (25 µmol m^{-2} s^{-1}).

4.2.10.2 Effect of explant source

Immature seeds of the cv. Honey Dew were germinated aseptically as described earlier in Chapter 3. Hypocotyl (1-2 mm), cotyledons (detached at the point of joining to the embryo axis), roots and shoot tip (with 1-2 leaves) from 10-day-old seedlings and leaflets from 15-day-old seedling were screened for their embryogenic potential. The leaf explants were separated from the seedling and the explants were prepared by discarding

the petiole and leaf apex. The leaf was cut along the midrib and the portion with midrib was made into pieces of 2-3 mm^2 and cultured. The explants were cultured on MBG basal medium supplemented with 3.0 mg/l 2,4,5-T. Cultures were incubated as above and induction of embryos was recorded after 4 weeks.

4.2.10.3 Effect of carbohydrate source

Five carbohydrate sources, like sucrose, glucose, maltose, glycerol and fructose were used to evaluate induction of somatic embryos from immature explant in cv. Honey Dew. The cultures were incubated as above for 4 weeks.

4.2.10.4 Effect of culture condition (light v/s dark incubation)

The immature embryos of cv. Honey Dew were inoculated on MBG basal medium supplemented with 3.0 mg/l 2,4,5-T. The cultures were incubated under different culture conditions like 16 h photoperiod light, 24 h photoperiod light and under dark incubation for 4 weeks.

4.2.10.5 Effect of gelling agent for immature explant

Different types of solidifying agents like agar 0.75% (Qualigens), phytagel 0.2% (Sigma), Gelrite 0.2% (Kelco) and agarose 0.75% (SRL) were tested to see their effect on somatic embryo induction in MBG basal medium supplemented with 3.0 mg/l 2,4,5-T and 3% sucrose. Influence of gelling agent was recorded after 4 weeks of incubation.

4.2.11 Parameters studied on mature embryo explant

Various parameters like basal medium, pH of the medium, AgNO3, temperature and physical state of the medium (solid/liquid) were evaluated to enhance the frequency of induction of somatic embryos. The cv. Co-2 has been used in all this case.

4.2.11.1 Effect of different basal medium

Five different basal medias such as MS, B5, 1/2 MS, Whites and MS basal media +B5 vitamin supplemented with 2 mg/l glycin (hereafter will be referred to as MBG) with 3% sucrose and 0.75% agar were used to test the induction of somatic embryogenesis in the cv. Co-2**.** The cultures were incubated at 25±2 ^{0}C for 4 weeks with a 16 h photoperiod under cool white fluorescent light (25 µmol m^{-2} s^{-1}).

4.2.11.2 Effect of pH of the medium

Different pH (5.5,5.8,6.0,6.5,7.0,7.5,8.0) value of the medium was tested to see their effect in inducing somatic embryo of mature embryo explant of papaya. All the

explants were inoculated in MBG medium supplemented with 3.0 mg/l 2,4,5-T with filter paper support and incubated as above for 4 weeks.

4.2.11.3 Effect of temperature

Explants were inoculated in MBG medium supplemented with 3.0 mg/l 2,4,5-T with filter paper bridge and incubated as above for 4 weeks in three different temperature conditions (25±2 ^{0}C, 27 ^{0}C, 30 ^{0}C with a 16 h photoperiod under cool white fluorescent light (25 µmol m^{-2} s^{-1}).

4.2.11.4 Effect of Silver nitrate ($AgNO_3$)

Silver nitrate ($AgNO_3$) in three different concentrations (1,3 and 5 mg/l) were used to supplement the MBG basal medium containing 3.0 mg/l 2,4,5-T and 3.0% sucrose to see the effect of $AgNO_3$ on induction of globular stage somatic embryos from mature embryo axes explant**.**

4.2.11.5 Effect of physical state of medium

Solid and liquid medium was also tested for their effect to induce somatic embryos from mature embryo axis of papaya. Number of globular somatic embryos per explant in both the types of media was also counted.

4.3 Results and Discussion

4.3.1 Immature embryo explant

Immature embryos of many plant species can be induced to undergo somatic embryogenesis and the auxin 2,4-D plays a significant role in the process (Ammirato 1983). This view was further documented with the report of Williams and Maheshwaran (1986) when they could induce somatic embryogenesis in immature embryos of 20 dicotyledonous species. In papaya, ovular tissues are considered to be the best source of regenerable cultures (Fitch 1990). Regeneration of papaya from immature ovular tissues are reported earlier (Fitch 1990; Castillo *et al.* 1998a) in exotic varieties. Here, we have studied the induction of somatic embryos in some commonly grown cultivars in India and various factors affecting its frequency.

4.3.1.1 Effect of growth regulators on induction of somatic embryogenesis

Basal medium supplemented with various auxins was reported to induce somatic embryogenesis in various crops (Sagare *et al.*1993; Hazra *et al.* 1989; Kysely & Jacobsen 1990). In papaya also, somatic embryos were induced from immature embryos with the

use of auxin (Fitch 1990; Monmerson *et al*. 1995; Castillo *et al*. 1998b). In the present investigation, various auxins at different concentrations, 2,4-D (1-25 mg/l), 2,4,5-T (0.5-20 mg/l), Picloram (0.5-10.0 mg/l), Dicamba (1.0-15.0 mg/l) and combination of 2,4-D (1-5mg/l) and 2,4,5-T (1 and 2mg/l) were supplemented in MS basal medium for the induction of somatic embryogenesis on three cultivars Honey Dew, Co-2 and Washington (Table 4.1). Among the auxins tested, 2,4-D and 2,4,5-T could induce somatic embryos in all the three cultivars tested while the response of Picloram and Dicamba was restricted to Honey Dew and Co-2 cv.. Non embryogenic friable callus has been observed in cv. Washington. When 2,4-D was used as hormonal supplement, best result in terms of explant response and globular embryos per explant was obtained at 2.0 mg/l, in all the cultivars. The explant response was observed to be 86% and 28 embryos per explant in cv. Honey Dew, 93% and 21 embryos / explant in Washington and 76% and 23 embryos / explant in cv. Co-2 (Table 4.2).

Table 4.1 Effect of different growth regulators on somatic embryogenesis of 3 cultivars of papaya

Auxin (mg/l)	Response in cultivars		
	Honey Dew	Washington	Co-2
2,4-D(1.0)	SE	SE	SE
2,4-D(2.0)	SE	SE	SE
2,4-D(5.0)	SE	SE	SE
2,4-D(10.0)	SE	SE	SE
2,4-D(15.0)	SE	SE	SE
2,4-D(25.0)	C	SE	SE
2,4-D(30.0)	C	NR	NR
2,4,5-T(0.5)	SE	SE	SE
2,4,5-T(1.0)	SE	SE	SE
2,4,5-T(2.0)	SE	SE	SE
2,4,5-T(3.0)	SE	SE	SE
2,4,5-T(5.0)	SE	SE	SE
2,4,5-T(8.0)	SE	SE	SE

2,4,5-T(10.0)	C	SE	C
2,4,5-T(20.0)	NR	NR	NR
Picloram (0.5)	SE	C	SE
Picloram (1.0)	SE	C	SE
Picloram (2.0)	SE	C	SE

Table 4.1 Continued..

Table 4.1 (Contd..)

Auxin (mg/l)	**Response in cultivars**		
	Honey Dew	**Washington**	**Co-2**
Picloram (3.0)	SE	C	SE
Picloram (5.0)	SE	C	SE
Picloram (10.0)	SE	NR	SE
Picloram (15.0)	NR	NR	NR
Dicamba (0.5)	NR	NR	NR
Dicamba (1.0)	NR	SE	SE
Dicamba (2.0)	NR	SE	SE
Dicamba (5.0)	NR	SE	SE
Dicamba (7.0)	NR	SE	SE
Dicamba (1.0)	NR	SE	SE
Dicamba (15.0)	NR	SE	SE
Dicamba (20.0)	NR	NR	NR
2,4-D(1.0)+2,4,5-T(1.0)	C	C	C
2,4-D(2.0)+2,4,5-T(1.0)	C	C	C
2,4-D(5.0)+2,4,5-T(1.0)	C	C	C
2,4-D(1.0)+2,4,5-T(2.0)	C	C	C
2,4-D(2.0)+2,4,5-T(2.0)	C	C	C
2,4-D(5.0)+2,4,5-T(2.0)	NR	NR	NR

SE-Somatic embryos; C-Callus; NR-Not responded

Table 4.2 Effect of 2,4-D on induction of somatic embryogenesis.

2,4-D concentration	% explant responded			Globular embryos per explant		
	Co-2	HD	WA	Co-2	HD	WA
Control	G	G	G	G	G	G
2	76.22±1.34 [a]	86.36 ± 6.94 [b]	93.61± 4.04 [a]	23.62± 1.25 [a]	28.33± 3.30 [b]	21.66± 5.50 [a]
5	58.51±4.79 [b]	66.47± 7.78 [a]	69.09± 4.00 [b]	16.33± 0.88 [b]	20.33± 3.68 [c]	14.00± 1.00 [b]
10	27.70±3.84 [c]	32.75± 3.26 [c]	36.54 ± 3.05 [d]	15.56± 1.84 [b]	20.33± 4.92 [c]	21.00± 1.00 [a]
15	15.0± 1.00 [d]	30.68 ± 1.63 [d]	48.58 ±1.52 [c]	12.33±1.52 [c]	14.33± 2.49 [a]	14.33± 3.78 [b]
25	07.0± 2.00 [d]	00.00± 0.00 [e]	17.36± 1.52 [e]	07. 0±1.0 0 [c]	00.00± 0.00 [d]	11.33± 3.51 [b]

MS medium without 2,4-D, served as control. Values with same letter (a-e) in a column are not statistically different at $P = 0.05$. G Germination

HD-cv. Honey Dew, Co-2-cv. Co-2 and WA-cv. Washington

However, cv. Honey Dew did not respond at the highest 2,4-D concentration (25 mg/l), like the other two cultivars. The three cultivars were found to vary in their embryogenic responses to the different concentrations of 2,4-D applied to the media. Similarly, when media was supplemented with 2,4,5-T at higher concentration (10 and 20 mg/l), no response was observed in Honey Dew and Co-2. The lowest response was observed in cv. Washington (1.46%) with 10mg/l of 2,4,5-T.The maximum response of in terms of percent induction of embryos was obtained with 3.0 mg/l of 2,4,5-T in all 3 cultivars being 79% in Honey Dew, 71% in Co-2 and 76% in Washington. Among all the concentrations tested, 3 mg/l of 2,4,5-T was found to be the optimum for maximum number of globular embryos per explant (Table 4.3). When Picloram was tested for somatic embryogenesis, maximum response was obtained in media supplemented with 1.0 mg/l Picloram (Table 4.4) in both Honey Dew and Co-2 cultivars. The overall explant response was found to be 58% and 26 embryos per explant in case of Honey Dew and 56% and 24 embryos per explant in case of Co-2. Our result does not exactly match with the earlier observation found in papaya (Fitch 1990). This variation might be due to the cultivar difference.

Table 4.3 Effect of 2,4,5 T on induction of somatic embryogenesis

2,4,5-T concentration	% explant responded			Globular embryos per explant		
	HD	Co-2	WA	HD	Co-2	WA
Control	G	G	G	G	G	G
0.5	08.33±1.52 [d]	03.66±1.53 [d]	04.93±0.40 [f]	10.66±2.30 [b]	11.00±1.00 [b]	14.00±1.00 [d]
1.0	51.00±3.60 [b]	39.66±3.05 [c]	41.20±1.15 [c]	16.66±2.51 [b]	22.66±3.00 [a]	16.4±0.56 [c]
2.0	66.66± 5.68 [b]	53.33±4.72 [b]	56.96±0.94 [b]	25.33±4.50 [a]	23.66±3.51 [a]	27.3±2.08 [b]
3.0	79.33± 5.03 [a]	71.00±3.60 [a]	76.73±1.10 [a]	29.33±3.21 [a]	27.66±4.04 [a]	33.00±4.35 [a]
5.0	40.66 ±2.51 [c]	41.00± 2.64 [c]	36.60±1.16 [d]	13.66±3.05 [b]	15.00±1.73 [b]	7.20±1.70 [e]
8.0	03.66 ±2.08 [d]	08.66 ± 1.53 [d]	05.48±0.61 [e]	04.33±4.04 [b]	04.66±2.08 [c]	6.73±1.10 [e]
10.0	0 [d]	0 [e]	1.46±0.64 [g]	0 [b]	0 [c]	1.90±0.96 [f]
20.0	0 [d]	0 [e]	0 [h]	0 [b]	0 [c]	0 [f]

MS medium without 2,4,5-T, served as control. Values with same letter (a-h) in a column are not statistically different at $P = 0.05$. G Germination

HD-cv. Honey Dew, Co-2-cv. Co-2 and WA-cv. Washington

Table 4.4 Effect of Picloram on induction of somatic embryogenesis in papaya

Picloram concentration	% explant responded		Globular embryos per explant	
	HD	Co-2	HD	Co-2
Control	G	G	G	G
0.5	42.18±1.91 [b]	40.62±2.13 [b]	15.83±1.04 [c]	14.73±2.33 [c]
1.0	58.29±2.06 [a]	56.66±3.05 [a]	26.73±2.54 [a]	24.05±0.78 [a]
2.0	32.33±1.85 [c]	30.55±1.50 [c]	21.44±1.70 [b]	19.33±2.08 [b]
5.0	14.11±1.16 [d]	13.10±0.84 [d]	12.03±1.78 [d]	10.66±0.57 [d]
10.0	03.44±0.76 [e]	02.66±0.58 [e]	1.55±0.38 [e]	1.22±0.19 [e]

MS medium without Picloram served as control. Values with same letter (a-e) in a column are not statistically different at $P = 0.05$. G Germination. HD-cv. Honey Dew, Co-2-cv. Co-2 and WA-cv. Washington

We have also tested the effect of Dicamba for inductinon of somatic embryogenesis in our study. MS basal medium supplemented with 5.0 mg/l Dicamba resulted maximum response (Table 4.5).

Table 4.5 Effect of Dicamba on induction of somatic embryogenesis in papaya

Dicamba concentration	% explant responded		Globular embryos per explant	
	WA	Co-2	WA	Co-2
Control	G	G	G	G
1.0	13.62±1.33 [c]	13.11±1.64 [d]	06.58±0.54 [f]	07.30±0.16 [c]
2.0	14.77±1.07 [c]	15.77±1.67 [c]	07.91±0.79 [b]	08.90±0.95 [b]
5.0	32.77±2.55 [a]	34.83±1.68 [a]	15.00±1.73 [a]	16.07±1.11 [a]
7.0	21.74±1.09 [b]	23.0±2.00 [b]	04.6±0.43 [a]	04.96±0.61 [d]
10.0	07.23±0.60 [d]	7.72±0.25 [c]	2.23±1.73 [e]	2.90±1.11 [f]
15.0	03.21±1.13 [e]	03.4±1.44 [f]	1.10±0.43 [f]	3.73±0.61 [e]

MS medium without Dicamba served as control. Values with same letter (a-f) in a column are not statistically different at $P = 0.05$. G Germination. HD-cv. Honey Dew, Co-2-cv. Co-2 and WA-cv. Washington

However, in all cases indirect embryogenesis was observed. Cultivar Washington responded with overall 32% induction and 15 embryos per explant while 34% induction of globular somatic embryos was observed in Co-2 with an average number of 16 embryos per explant. Plant genotype can be a critical factor for induction of somatic embryogenesis from tissues (Thorpe 1988; Parrott *et al.* 1995). Therefore, screening of genotypes is a prerequisite for intensive studies in somatic embryogenesis (Rangaswamy 1986). Considering this view, due to the availability and being popular cv., we have selected Honey Dew cv. for our further experiments on different parameters that affect somatic embryogenesis from immature embryo axes.

4.3.1.2 Induction of somatic embryos and their development to cotyledonary stage

Immature embryo of the cultivars Honey dew, Co-2 and Washington when cultured on different concentrations of auxins showed varied response. After 5 days of incubation the explant became swollen and cotyledons expanded. Within 3-4 weeks of culture on MS medium supplemented with the auxins (2,4-D, 2,4,5-T, Picloram and

Dicamba), globular somatic embryos were observed from the shoot pole region of the embryo axis explants. Somatic embryos first appeared as round and globular masses and were loosely attached to the shoot pole region (Fig. 4.2).

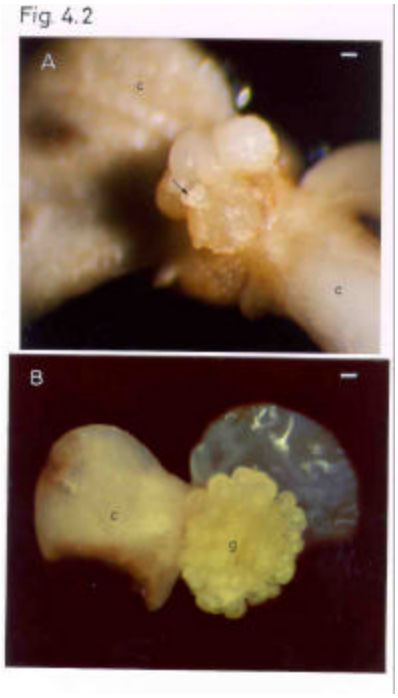


Fig. 4.2

A. Globular somatic embryos originating from shoot pole region of the immature embryos. Arrow indicates a single globular embryo, c-cotyledon. Bar=21.5 µm

B. Bunches of globular somatic embryos originating directly from the embryo explant after 3 weeks of incubation, c- cotyledon, g-globular embryo. Bar= 19µm

However, cotyledons or root pole of the explants were also able to produce embryos directly (Fig. 4.3). The developmental pattern of the somatic embryos was continuos and essentially asynchronous passing through various developmental stages such as heart, torpedo and cotyledonary structures (Fig. 4.4).

An established culture usually consists of somatic embryos having various developmental stages. Pale greenish or whitish embryos upon transfer to the induction medium produced mature or secondary somatic embryos from the hypocotyl or radical pole region of the primary embryos (Fig. 4.5A). While shoot tip region of the explant

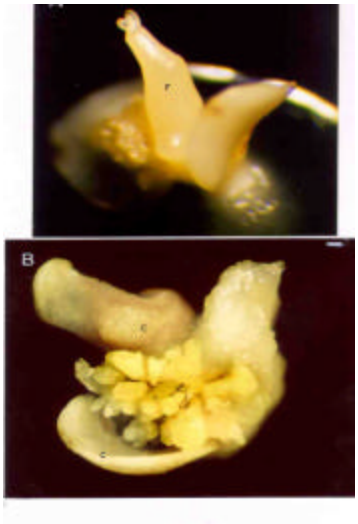


Fig. 4.3

A. Globular somatic embryos origination from root pole of the embryo explant, r-root. Bar=410µm

B. Somatic embryos originating from cotyledons of the embryo explant, c -cotyledons Bar=250µm

formed somatic embryos, the root pole region turned into brown callus or became swollen in appearance (Fig. 4.5B). Explants that did not initiate somatic embryos commonly developed unorganized friable callus and sometimes with adventitious roots.

With 2,4-D in the basal medium, direct somatic embryogenesis was observed (Fig. 4.2 B). Incubation of zygotic embryos on MS basal medium did not favour somatic embryogenesis rather resulted in their germination or remain quiescent. With 2,4,5-T and Picloram in the basal medium, somatic embryos originated both directly and also via an intermediary callus phase from the explant (Fig 4.6) .The type of regeneration response

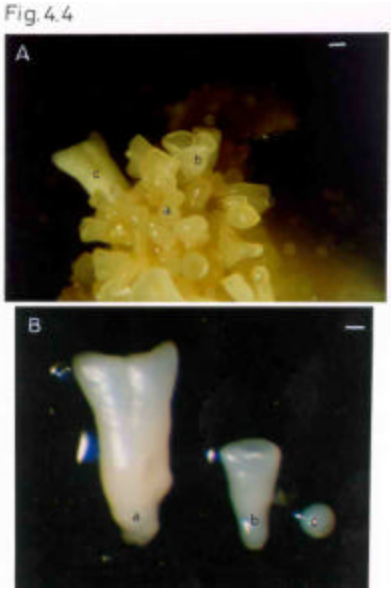


Fig. 4.4

A. Bunch of somatic embryos in various developmental stage, a-globular stage, b-torpedo stage , c- cotyledonary stage. Bar=2.14mm

B. Various stages of papaya somatic embryogenesis, a-late cotyledonary stage with root pole, b-torpedo staged embryo, c-single globular staged embryo.Bar=160µm

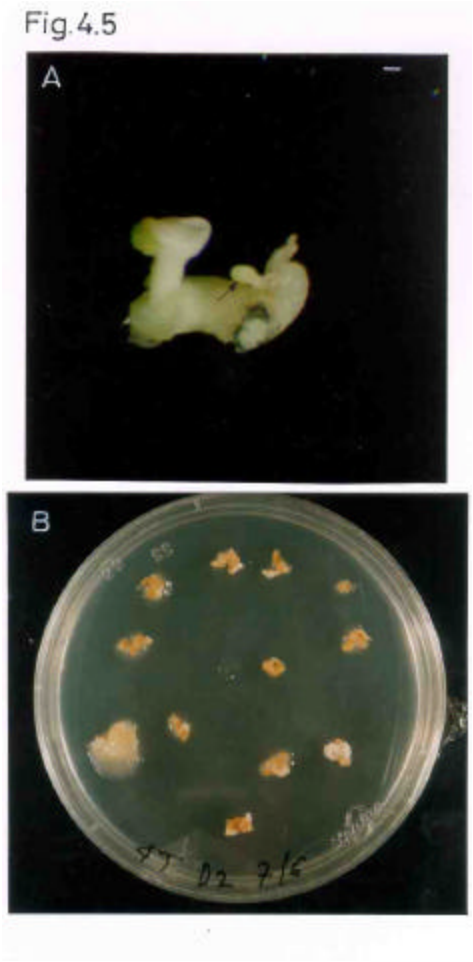


Fig. 4.5

A. Secondary embryo originating from the root pole of primary embryo, þprimary embryo. Arrow indicates secondary embryo. Bar=375 µm

B. Root poles of the embryo explant forming brown callus while shoot pole formed embryos

was found to be dependent on the concentration of the auxin used. Origin of the developed somatic embryos was confirmed by histological and scanning electron microscope studies (Fig. 4.7-4.9). Secondary somatic embryogenesis was also found to be a common occurrence. General observation was that lower concentration up to 3.0

mg/l of both 2,4,5-T and 2,4-D and also in Picloram (2.0 mg/l), explant favoured direct somatic embryogenesis whereas higher concentration tested lead to the formation of somatic embryos accompanied by callus proliferation (Fig. 4.10A).

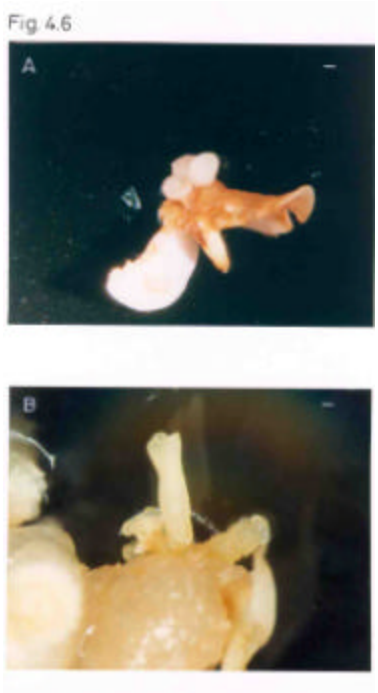


Fig. 4.6

A. Direct origin of the somatic embryos from the immature embryo explant. Bar=372 µm

B. Indirect and asynchronous development of the somatic embryos form immature embryo explant Bar=500 µm

However, with Dicamba in the basal medium, indirect somatic embryogenesis of the explant was observed at all concentration tested (Fig. 4.10 B).

Two hundred globular staged somatic embryos derived from the medium containing 2,4-D (2.0 mg/l) and two hundred embryos from medium with 2,4,5-T (3.0 mg/l) were sub cultured on the same medium in petridishes for their further development into cotyledonary stage somatic embryos (Fig. 4.11). After another 4-5 weeks, globular stage embryos passed through various stages of embryo development, such as torpedo and cotyledonary structures. In general, it took about 8-10 weeks to obtain cotyledonary structures starting from the initiation of the culture of explants in the induction medium. A large number of cotyledonary structures formed were found to be morphologically abnormal. Typical abnormalities include**:** missing of cotyledons, extra or fused cotyledons, or fusion of several embryos at their hypocotyl or overall grossly misshapen embryo morphology (Fig. 4.12). Many ~~a~~ times such structures failed to germinate with proper shoot apex, they produced only root. Similar type of abnormal embryo morphology was also observed earlier (Ozias- Akins 1989; Suhasini *et al.* 1996).

Table 4.6 Conversion of 2,4-D induced globular embryos into cotyledonary staged embryos

2,4-D concentration	% conversion of globular to cotyledonary stage somatic embryo		
	Co-2	HD	WA
2	23.66 ± 1.52 [c]	42.11 ± 4.49 [b]	41.50 ± 5.13 [a]
5	41.44 ± 0.76 [a]	43.58 ± 3.74 [a]	38.78 ± 1.15 [a]
10	32.11 ± 1.16 [b]	35.84 ± 2.49 [c]	22.65 ± 2.00 [b]
15	22.07 ± 1.00 [d]	17.15 ± 1.63 [d]	17.14 ± 3.78 [c]
25	3.51 ± 0.50 [c]	00.00 ± 0.00 [e]	14.28 ± 1.00 [c]

Values with same letter (a-e) in a column are not statistically different at *P* = 0.05. HD-cv. Honey Dew, Co-2-cv. Co-2 and WA-cv. Washington

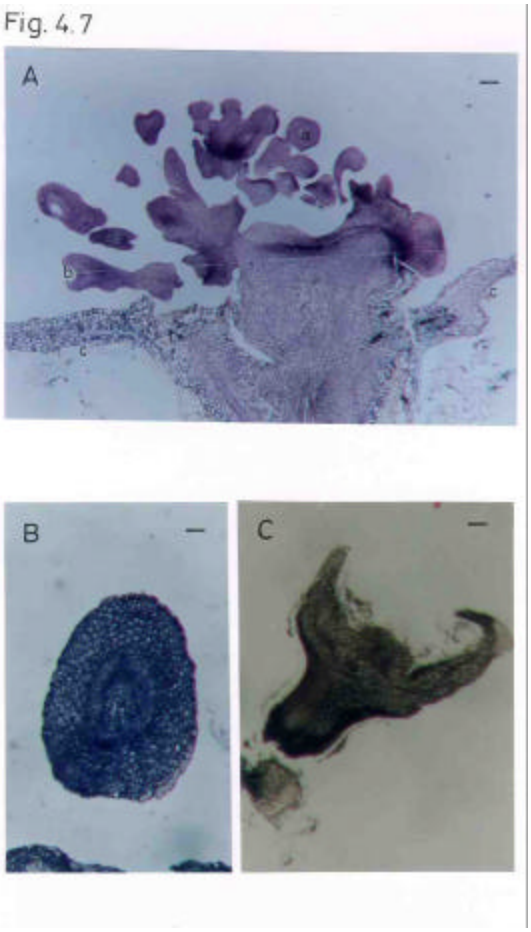


Fig. 4.7

A. Longitudinal section (LS) section of the immature embryo axis of cv. Honey Dew, passing through the shoot pole region after 5 weeks showing the origin of the somatic embryos at various developmental stages. a-globular embryo, b-cotyledonary staged embryo, c-cotyledons of the embryo explant. Bar=384µm

B. A single globular embryo originating directly from the embryo explant, c-cotyledon. Bar=125µm

C. Magnified view of a single heart shaped somatic embryo Bar=200µm

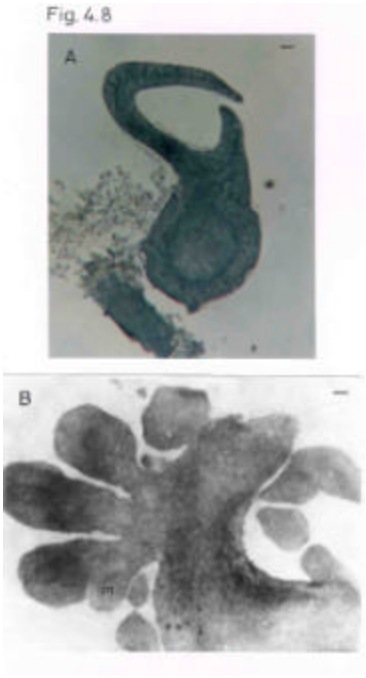


Fig. 4.8

A. Single cotyledonary staged papaya somatic embryo showing prominent vascular structure. Bar=485µm

B. Longitudinal section of immature embryo cotyledons showing the origin of both primary and secondary embryo, p-primary embryo, p1-seconadry embryo. Bar=190 µm

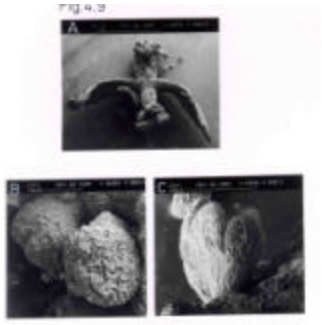


Fig. 4.9

A. Scanning electron micrograph study showing direct origin of the somatic embryos from the shoot pole region of the immature papaya embryo, e-embryos, c-cotyledons, r- root pole

B. Scanning electron micrograph study showing a single globular and an early heart shaped papaya somatic embryo. g-globular embryo, h-heart shaped embryo

C. Scanning electron micrograph study of a single cotyledonary staged somatic embryo, having multiple cotyledons

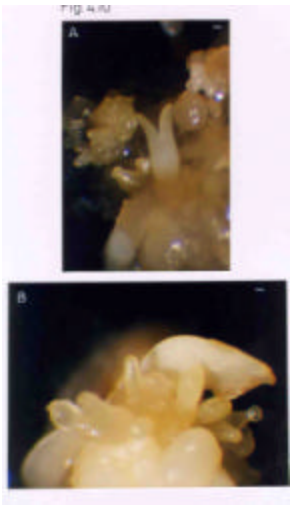


Fig. 4.10

A. Development of papaya somatic embryos accompanied by callus proliferation in presence of higher concentration of hormones (2,4,5-T,8.0 mg/l). Bar=200 µm

B. Indirect somatic embryogenesis obtained in presence of hormone Dicamba (2.0mg/l). Bar=960µm

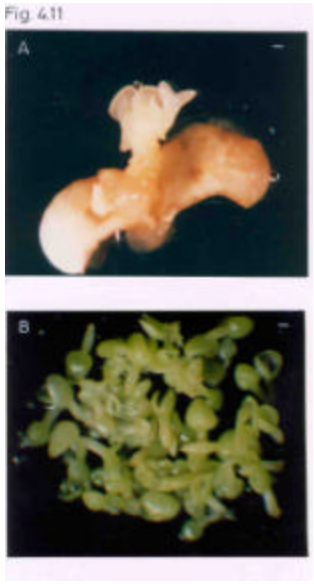


Fig. 4.11

A. Direct formation of cotyledonary staged papaya somatic embryos from immature embryo explant.Bar=263 µm

B. A number of loosely arranged late cotyledonary staged papaya somatic embryos.Bar=250µm

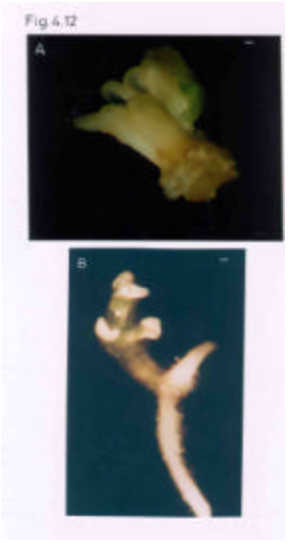


Fig. 4.12

A. Fused papaya somatic embryos obtained in cv. Honey Dew when cultured in 8.0mg/l 2,4,5-T.Bar=133 µm

B. Abnormally developed papaya somatic embryo generated seedling with improper shoot meristem development.Bar=120µm

Table. 4.7 Conversion of 2,4,5-T induced globular embryos into cotyledonary stage embryos

2,4,5-T concentration	% conversion of globular to cotyledonary stage somatic embryo		
	HD	Co-2	WA
0.5	23.33±1.53 [c]	12.00±2.00 [c]	15.88±0.83 [e]
1.0	42.33±4.50 [b]	17.66±1.15 [c]	23.53±1.50 [c]
2.0	52.33±1.53 [a]	31.66±4.16 [b]	42.66±2.08 [a]
3.0	53.33±1.53 [a]	45.66±3.21 [a]	36.00±1.00 [b]
5.0	38.33±3.51 [b]	33.00±4.59 [b]	22.07±0.89 [d]
8.0	07.66±4.04 [d]	15.66±5.03 [c]	02.89±0.30 [f]
10.0	0 [e]	0 [d]	01.06±1.00 [g]
20.0	0 [e]	0 [d]	0 [g]

Values with same letter (a-g) in a column are not statistically different at $P = 0.05$. HD-cv. Honey Dew, Co-2-cv. Co-2 and WA-cv. Washington

When 2,4-D was used in the medium, maximum conversion of globular to cotyledonary stage embryos was achieved in cv. Co-2 (41.44%) and in Honey Dew (43.58%) with 2,4-D concentration at 5mg/l, whereas 41.50% conversion rate was observed in cv. Washington with 2 mg/l of 2,4-D in the media (Table 4.6). Similarly, with 2,4,5-T in the basal medium maximum conversion was obtained with Honey Dew (53.33%) and Co-2 (45.66%) at a level of 3.0mg/l of 2,4,5-T, whereas cv. Washington responded best (42.66%) with 2 mg/l of 2,4,5-T in the medium (Table 4.7).

4.3.1.3 Maturation of somatic embryos

Maturation of the somatic embryos is marked by expansion of cells and accumulation of storage reserves (Raghavan 1986). The term maturation used here, denotes the development of cotyledonary stage somatic embryos into distinct bipolar structures with well-defined root and shoot pole and with expanded cotyledons. The normal cotyledonary stage somatic embryos derived from MS basal medium supplemented with 2,4-D (5.0 mg/l) and 2,4,5-T (3.0 mg/l) when cultured on MS basal medium containing 3% sucrose, 0.75% agar and 0.1 mg/l ABA, developed mature somatic embryos after 5-7 days of incubation (Fig. 4.13A). Maturation of somatic embryos was also tested in the same induction medium (MS basal medium with 2.0 mg/l of 2,4-D or 3.0 mg/l of 2,4,5-T) which served as control medium in this experiment. Mature somatic embryos have well developed root and shoot pole (Fig. 4.13 B). Fifty cotyledonary stage embryos of 3 varieties from 2,4-D media and fifty cotyledonary stage embryos of 3 varieties from 2,4,5-T media were kept separately in medium with ABA for maturation. On an average, 64% of embryo maturation was observed in Honey Dew, 54% in Co-2 and 62% in Washington in medium supplemented with ABA (0.1mg/l). In this case, embryos were induced from 2,4-D supplemented media. Whereas embryos induced from media supplemented with 2,4,5-T matured on an average at 68% in Honey Dew, 56% in Co-2 and 60% in Washington when transferred to media containing ABA (0.1mg/l). However, in the 2,4-D induction medium (control media without ABA),

maturation rate was 20% in Honey Dew, 16% in Co-2 and 28% in Washington. Remaining embryos turned necrotic or formed callus. The beneficial effect of ABA on conversion of somatic embryogenesis has been reported in papaya (Castillo *et al*. 1998a; Monmerson *et al*. 1995) and other crops (Roberts *et al*. 1990; Suhasini *et al*.1996). ABA prevents precocious germination of the cotyledonary staged embryos. Precocious or premature development, particularly germination is a major problem during somatic embryo development and this is controlled either by increasing osmolarity of the maturation medium with additional sucrose (Carman 1989) or by incorporating ABA in to the medium (Ammirato 1974). ABA may be necessary during embryogenesis to initiate the synthesis of storage proteins and proteins involved in desiccation tolerance (Galau *et al*. 1990). ABA was also reported to influence development of carrot somatic embryos and in particular affected their capacity to develop functional shoot meristem (Nickle and Yeung 1994).

4.3.1.4 Germination and conversion of somatic embryos

The term germination denotes the elongation of the primary root and conversion refers to the development of plantlets with a well established root system and shoot with at least the first pair of leaves (Mathews *et al*. 1993). For germination, 25 matured embryos of each variety from 2,4-D containing induction medium and 25 embryos of each of 3 varieties from 2,4,5-T induction medium were shifted to various media formulations to encourage their germination (Table 4.8). Freshly transferred somatic embryos started expansion within 5-10 days after transfer and germinated within 3 weeks of incubation, with adventitious root initiation and shoot extension (Fig. 4.14).

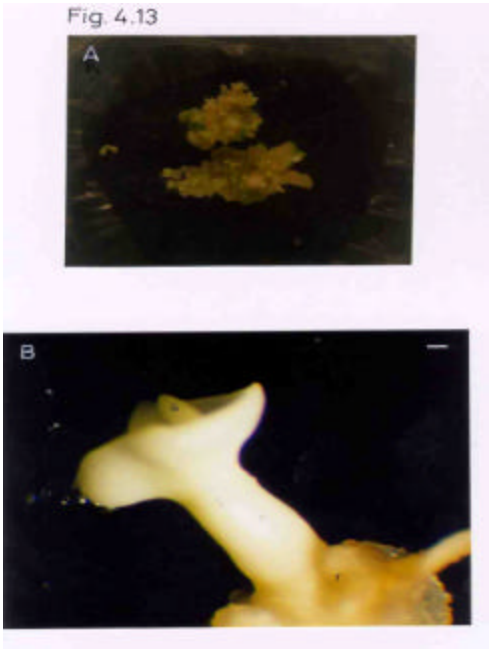


Fig. 4.13

A. A group of mature papaya somatic embryos cv. Honey dew
B. Single mature somatic embryo with developing shoot and root pole. s-shoot pole, r-root pole cv. Honey dew. Bar=100µm

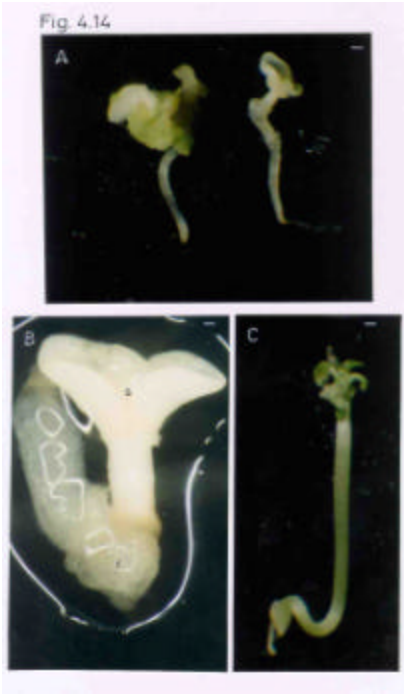


Fig.4.14

A. Germination of papaya somatic embryos cv. Honey DewBar=153 µm
B. Mature germinating cotyledonary stage papaya somatic embryo showing properly devloped shoot and root pole. s-shoot pole, r-root cv. Washington.Bar=111µm
C. A papaya somatic embryo derived germinated seedling cv. Co-2.Bar=136µm

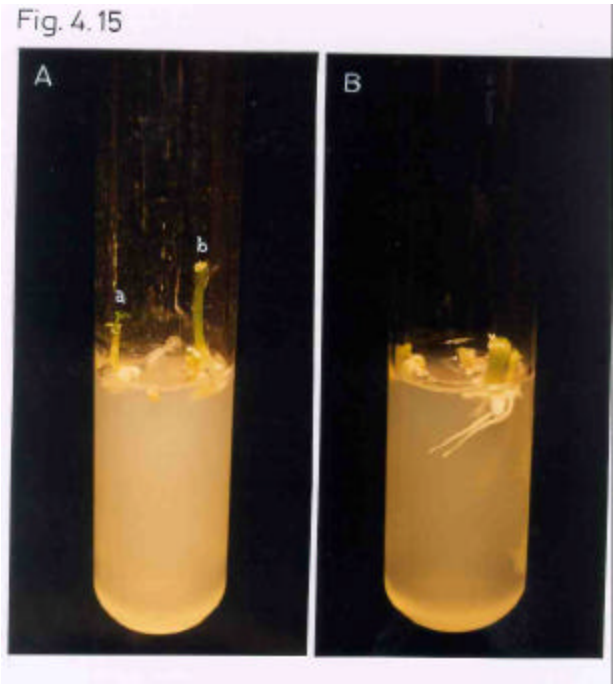


Fig. 4.15

A. Abnormal germination of papaya somatic embryos a-shoot development without any root formation, b- seedling with improper shoot and root development

B. Germinated seedling with root development but improper shoot development

Table 4.8 Germination of cotyledonary stage somatic embryos in presence of different growth regulators

Germination media	% germination in 2,4-D media			% germination in 2,4,5-T media		
	HD	WA	Co-2	HD	WA	Co-2
MS	58.66±2.30 [c]	60.33±2.30 [c]	65.33±2.3 0 [b]	71.33±2.08 [a]	54.60±2.30 [b]	59.33±1.50 [a]
MS+ABA (0.1mg/l)	66.66±2.30 [b]	63.66±4.00 [b]	38.66±6.11 [c]	41.33±2.30 [c]	41.30±2.30 [c]	49.33±2.30 [c]
MS+BAP (1mg/l)	00.00 [d]	4.00±2.30 [d]	00.00 [d]	000.00 [e]	5.33±2.30 [e]	0 0.00 [d]
MS+Zeatin(1mg/l)	00.00 [d]	00.00 [e]	00.0 0 [d]	16.33±2.51 [d]	10.6±2.30 [d]	00.00 [d]
MS+GA_3 (1mg/l)	70.66±2.30 [a]	72.0±4.00 [a]	73.33±2.30 [a]	61.33±2.08 [b]	73.3±2.30 [a]	54.66±1.15 [b]

Values with same letter (a-e) in a column are not statistically different at P = 0.05. HD-cv. Honey Dew, Co-2-cv. Co-2 and WA-cv. Washington

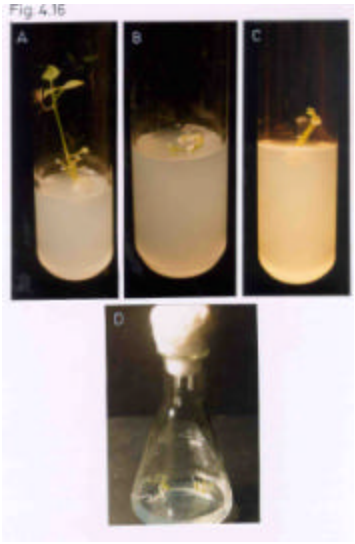


Fig.4.16

A. Papaya somatic embryo germination in presence of GA_3 (1.0 mg/l) in the germination medium (embryo developed from MS basal medium + 2,4-D, 2.0 mg/l cv. Washington
B. Papaya somatic embryo germination in presence of growth regulator free basal medium (embryo developed from media supplemented with 2,4,5-T, 3.0 mg/l) cv. Honey Dew
C. Papaya somatic embryo germination in growth regulator free basal medium cv. Co-2

The percentage of embryo germination did not increase upon extended incubation. Many well formed embryos failed to germinate mainly due to malformation of the epicotyl pole or abnormal development of shoot apex (Fig. 4.15). Embryos originating from 2,4-D medium, germinated maximum in presence of GA_3 (1.0 mg/l), having 70% in Honey Dew, 72% in Washington and 73% in Co-2 (Fig. 4.16A). Embryos originating from 2,4,5-T medium, germinated maximum in growth regulator free medium (Fig. 4.16 B), having 71% germination in Honey Dew and 59% in Co-2 (Table.4.8). The somatic embryos of cv. Washington germinated maximum (73%) in presence of GA_3. Although growth regulator free basal medium can support somatic embryo germination (Litz and Conover 1983) (Fig. 4.16 C), positive effect of GA_3 in embryo germination was also reported earlier (Chang and Hsing 1980). Fully germinated plantlets were grown on phytohormone free MS basal medium, for vigorous root and shoot growth (Fig. 4.17). Regenerated plantlets were morphologically similar to seed derived plants.

4.3.1.5 Transfer of plantlets to soil

Plant lets when attained a height of 3-5 cm were washed off media with deionized water, dipped in 1% Bavistin (fungicide) and were transferred to pots. Thereafter, 30

plantlets (10 of each variety) were transferred to pots (8 cm) with a potting mixture of sand, soil and farm yard manure (1:1:1 w/w). After 30 days of transfer to pots, 40% of the plantlets survived. Thus 12 plants (6 from Honey Dew, 4 from Co-2 and 2 from Washington) (i.e. 60% in Honey Dew, 40% in Co-2 and 20% in Washington) could be well established in the pots after hardening. Later, these plantlets were planted outside in the field. The growth of the plantlets was found to be normal (Fig. 4.18).

Fig. 4.17

A. Papaya somatic embryo derived germinated seedling originating from 2,4-D (2.0 mg/l) supplemented MS basal medium, cv. Washington

B. Papaya somatic embryo derived germinated seedling originating from 2,4,5-T (3.0 mg/l) supplemented MS basal medium, cv. Co-2

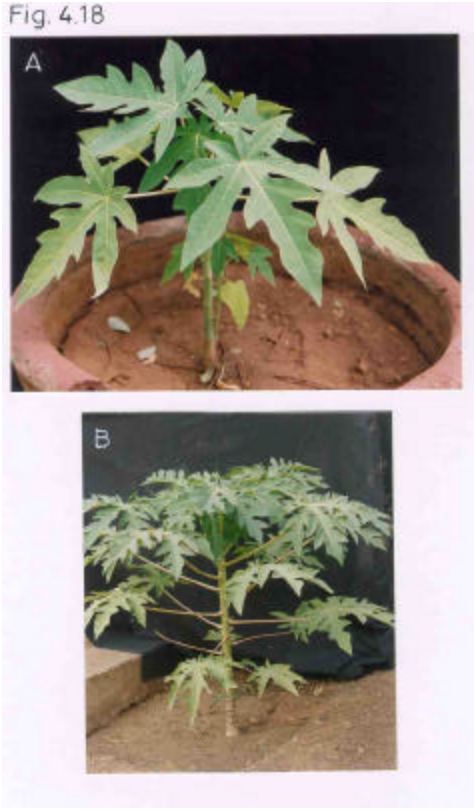


Fig. 4.18

A. Two months old somatic embryo derived seedling cv. Honey Dew in pot

B. Four months old somatic embryo derived seedling cv. Washington in field

4.3.2 Parameters studied in immature embryo

Several factors were found to influence the somatic embryogenesis of papaya (Huang *et al*. 1996). Therefore, **v**arious parameters were studied in order to standardize the somatic embryogenesis system in Indian cultivars. As the cv. Honey Dew is quite popular in the market and also could produce maximum cotyledonary stage embryos (53%) when induced on 2,4,5-T (3.0 mg/l) medium, hence, we selected the variety Honey Dew for our further parameter studies. The following studies were carried out keeping the cultivar (Honey Dew) and the induction medium (MS basal medium supplemented with 3.0mg/l 2,4,5-T, 3% sucrose and 0.75% agar) as constant.

4.3.2.1 Effect of basal media

In vitro growth of papaya tissues was found to be affected by various media constituents (Medora *et al.* 1984). Basal media such as MS, B5, 1/2 MS, Whites, MBG were tested in presence of 3.0 mg/l 2,4,5-T and 3% sucrose. MS media was found to be the best medium as it supported 79% induction and 29 embryos per explant followed by MBG and B5 basal media (Table-4.9). White's medium favoured least response (26% response) with on an average 1.9 embryo per explant while 1/2 MS failed to support the induction of somatic embryogenesis from the explant.

Although a wide range of basal medium can be used for the induction of somatic embryogenesis, over 70% of the successful cases used MS salts and its derivatives (Thorpe 1988). These media contain high levels of salts and both NH_4^+ and NO_3^-, in the process of somatic embryo induction (Thorpe 1988). As the MS basal media elicited best response, this medium was used for all the future experiments.

Table 4.9 Effect of basal media on somatic embryo induction in papaya.

Basal media	% induction of embryos	Globular embryos per explant
MS	79.33±5.03 [a]	29.33±3.21 [a]
MBG	50.00±1.00 [b]	12.06±2.00 [b]
B5	30.00±1.00 [c]	04.13±0.60 [c]
Whites	26.60±1.15 [d]	1.90±0.96 [d]
½ MS	NR	NR

Values with same letter (a-d) in a column are not statistically different at $P = 0.05$.

NR-Not responded

4.3.2.2 Effect of gelling agent

Gelling agents used to solidify plant growth media contain considerable amount of mineral nutrients which can effect plant growth (Debergh 1983) by affecting the availability of nutrients. Among the different types of gelling agent tested (agar 0.75%, phytagel 0.2%, Gelrite 0.2% and agarose 0.75%) in our experiment, normal agar evoked best response with 79% induction and 29 embryos per explant (Table 4.10). Explants cultured on media solidified with gelrite did not induce somatic embryos. Therefore, agar was used as solidifying agent in further experiments.

Table 4.10 Effect of gelling agent on somatic embryo induction in papaya

Gelling agent	% induction of globular embryo	Globular embryos per explant
Agar	79.33±1.15 [a]	29.33±1.15 [a]
Phytagel	43.07±1.11 [c]	11.74±1.40 [c]
Agarose	50.40±0.52 [b]	13.77±1.57 [b]
Gelrite	NR	NR

Values with same letter (a-c) in a column are not statistically different at $P = 0.05$.

NR-Not responded

4.3.2.3 Effect of culture condition (dark v/s light incubation)

Although cultures kept in all the three conditions could induce embryogenesis, the percentage induction of embryos was best in dark condition (79%) followed by 16 h light (49%) (Table 4.11). Embryos induced in continuos light were found to be more green in colour than those produced from dark or 16 h photoperiod condition.

The culture environment was found to influence the somatic embryogenesis process. Somatic embryogenesis can occur under a variety of light and dark conditions but in general, darkness may be better (Thorpe 1988). The requirement of course varies with the species.

Table 4.11 Effect of culture condition on somatic embryo induction in papaya

Culture condition	% induction of embryos	Globular embryos per explant
Dark	79.33±1.15 [a]	29.33±1.15 [a]
16 h light	49.96±1.00 [b]	14.17±0.85 [b]
24 h light	40.76±1.56 [c]	7.07±0.89 [c]

Values with same letter (a-c) in a column are not statistically different at $P = 0.05$.

NR-Not responded

4.3.2.4 Effect of carbohydrate source

In our experiments, when different carbohydrate sources were compared, it was found that sucrose supported maximum number of embryos per explant (29) followed by

maltose (25 embryos), fructose (13 embryos) and glucose (8 embryos) (Table 4.12). Glycerol did not support induction of somatic embryogenesis. In *in vitro* condition the autotrophic growth of plant tissues are hampered. To provide additional energy, carbohydrates are added (Kozai 1991; Leifert *et al*. 1995). Carbohydrates also act as osmotic agent in tissue culture (Thorpe 1982). Sucrose is the most common carbohydrate used in *in vitro* studies. This may be because carbohydrate is transported inside plant tissue as sucrose and the tissues have inherent capacity for uptake transport and utilization of sucrose (Eapen and George 1993). However, other sugars have also been reported being suitable for some species (Genga & Allavena 1991). Glucose has been found to be effective for *phaseolus coccineus* (Genga & Allavena 1991) while maltose was preferred in alfalfa (Strickland *et al*. 1987).

Table 4.12 Effect of carbon source on somatic embryo induction in papaya

Carbon source	% induction of embryos	Globular embryos per explant
Sucrose	79.33±1.15 [a]	29.33±1.15 [a]
Maltose	25.48±0.90 [b]	13.36±1.36 [b]
Fructose	13.78±1.40 [c]	04.51±0.83 [c]
Glucose	08.07±0.89 [d]	02.77±0.38 [d]
Glycerol	NR	NR

Values with same letter (a-d) in a column are not statistically different at $P = 0.05$.

NR-Not responded

4.3.2.5 Effect of explant source

In an early report in papaya tissue culture study (Miller and Drew 1990), type of explant was found to affect the regeneration response. Somatic embryos were also obtained from cotyledon and hypocotyl explants of cv. Honey Dew when cultured on MS basal medium supplemented with 3.0 mg/l 2,4,5-T containing 0.75% agar and incubated in dark for 10 weeks (Table 4.13). Globular embryos were found to arise directly or indirectly from cotyledon explant and from callus derived on the surface of hypocotyls. Embryo axes served as control in the experiment.

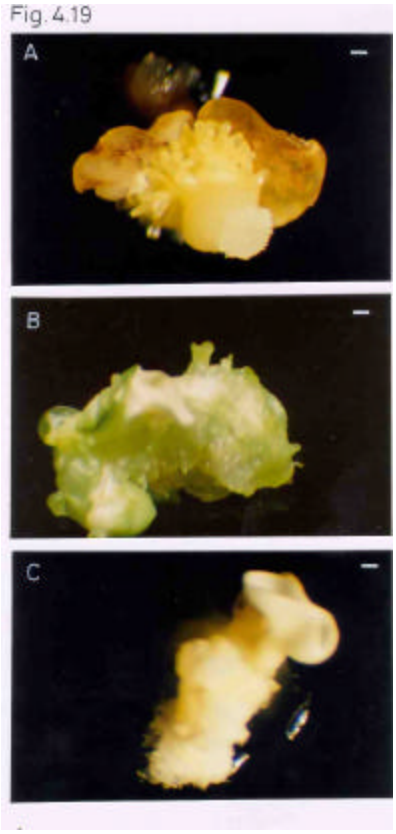


Fig. 4.19

A. Papaya somatic embryos developing from the embryo axes region of the immature embryo explant, cv. Honey Dew. Bar=312 µm

B. Somatic embryos developing from the cotyledons of immature embryo explant, cv. Honey Dew. Bar=333 µm

C. Somatic embryos developing from the hypocotyl region of the seedling explant, cv. Honey Dew. Bar=333 µm

Table 4.13 Effect of explant source on somatic embryo induction in papaya

Explant	% induction of embryo	Globular embryos per explant
Embryo axes	79.33±1.15 [a]	29.33±1.15 [a]
Cotyledon	03.02±0.12 [b]	04.77±0.69 [b]
Hypocotyl	01.06±0.11 [c]	01.06±1.00 [c]
Leaf	NR	NR

Values with same letter (a-c) in a column are not statistically different at $P = 0.05$.

NR-Not responded

Among the four types of explants used, shoot apice region of the embryo axes produced maximum percent induction of somatic embryos (79%) whereas hypocotyl sections produced the least (1%) response (Fig. 4.19). The development of globular stage somatic embryos derived from cotyledon or hypocotyl explants into cotyledonary stage somatic embryo is same as that observed from embryo axes derived somatic embryos.

4.3.3 Mature embryo explant

Reports on papaya somatic embryogenesis was limited to regeneration from immature embryo explants. Plant regeneration from immature explants have many constraints mainly the non availability of plant material through out the year and difficulty in obtaining the plant material at the right developmental stage (Baker and Wetzstein 1992; Malik and Saxena 1992). In addition, successful induction of somatic embryogenesis from immature explants relied on a number of factors such as size of embryo (Hazra *et al*. 1989) and the orientation of the cotyledonary explants on the culture medium (Ozias-Akins 1989). This prompted us to evaluate mature embryos for induction of somatic embryogenesis.

4.3.3.1 Effect of various auxins on somatic embryo induction from mature embryo axis

Based on our work on immature embryos of papaya, MS basal medium was selected and the auxins 2,4-D (1-5 mg/l), 2,4,5-T (1-5 mg/l), Picloram (1-10mg/l), Dicamba (1-10mg/l) were tested for inducing somatic embryos from mature embryos of Honey Dew and Co-2 cv. of papaya. Effects of all these hormones were tested in liquid (as liquid medium was found to produce better response in other experiments, described in chapter 5.) media for embryo induction (Table 4.14). Unlike immature embryos, only lower concentration of 2,4-D and 2,4,5-T responded in inducing somatic embryos from the explant (Fig. 4.20 A,B). Somatic embryos originated from the cotyledonary region of the explant. Explant that did not produce somatic embryos responded by producing loose friable non embryogenic callus (Fig. 4.20 C).

Table 4.14 Effect of growth regulators on somatic embryo induction from mature embryo axes

Growth regulators	Honey Dew	Co-2

2,4-D (1)	SE	SE
2,4-D (2)	SE	SE
2,4-D (3)	SE	SE
2,4-D (5)	C+SE	C+SE
2,4-D (10)	C	C
2,4,5-T (1)	SE	SE
2,4,5-T (2)	SE	SE
2,4,5-T(3)	SE	SE
2,4,5-T(5)	C+SE	C+SE
2,4,5-T(10)	NR	NR
Picloram(1)	C	C
Picloram(2)	C	C
Picloram(5)	NR	NR
Picloram(10)	NR	NR
Dicamba(1)	NR	NR
Dicamba(2)	NR	NR
Dicamba(5)	NR	NR
Dicamba(10)	NR	NR

SE-Somatic embryos; C-Callus; NR-Not responded

Table 4.15 Effect of 2,4-D on somatic embryogenesis of papaya from mature embryo axes in cultivars Honey Dew and Co-2

2,4-D	**Honey Dew**		**Co-2**	
	% Induction of embryos	**Globular embryos per explant**	**% Induction of embryos**	**Globular embryos per explant**
Control	G	G	G	G
1	09.66±2.08 [c]	09.21±0.29 [b]	12.40±2.50 [c]	10.85±1.87 [c]
2	35.51±0.71 [a]	16.44±1.50 [a]	42.37±2.57 [a]	18.11±1.83 [a]
3	23.28±1.70 [b]	13.40±2.12 [a]	24.51±3.78 [b]	14.40±0.52 [b]
5	07.07±1.40 [d]	05.16±2.46 [c]	09.53±1.26 [c]	04.5±1.32 [d]

MS medium without 2,4-D served as control. Values with same letter (a-d) in a column are not statistically different at $P = 0.05$. G Germination

HD-cv. Honey Dew and Co-2-cv. Co-2

Mature embryos of cv. Honey Dew and Co-2 when cultured on MS basal medium supplemented with various concentrations of 2,4-D and 2,4,5-T produced globular embryos directly or indirectly from the embryo axes. A varied response in percentage induction of somatic embryogenesis has been observed in media supplemented with various concentration of 2,4-D (Table 4.15). On an average, 7-35% explant responded globular somatic embryo in Honey Dew and 9 to 42% in Co-2 when cultured on media containing 2 mg/l 2,4-D. This concentration has favoured the highest percent induction and maximum number of globular embryos per explant in both the cultivars. Number of globular somatic embryos per explant varied from 5-16 in Honey Dew and 4.5 to 18 in Co-2 depending on the 2,4-D level used in the media.

Table 4.16 Effect of 2,4,5-T on induction of somatic embryogenesis from mature papaya embryo axes in cultivars Honey Dew and Co-2

2,4-T	Honey Dew		Co-2	
	% embryo induction	Globular embryos per explant	% embryo induction	Globular embryos per explant
Control	G	G	G	G
1	10.11±1.92 [c]	11.36±1.51 [b]	11.22±1.07 [c]	09.00±1.00 [c]
2	21.77±3.28 [b]	21.0±1.00 [a]	24.55±1.91 [b]	13.85±1.61 [b]
3	36.44±1.50 [a]	14.74±0.4 [b]	43.83±1.68 [a]	25.88±1.01 [a]
5	05.92±0.55 [d]	05.49±0.5 [d]	13.51±1.40 [c]	7.66±2.08 [c]

MS medium without 2,4,5-T served as control. Values with same letter (a-d) in a column are not statistically different at $P = 0.05$. G Germination

HD-cv. Honey Dew and Co-2-cv. Co-2

When 2,4,5-T was used in the media for induction of somatic embryogenesis, the percentage of induction ranged from 5 to 36% in Honey Dew and 11 to 43% in Co-2 (Table 4.16). In both the cultivars, highest percentage induction was observed in media supplemented with 3.0 mg/l 2,4,5-T (Honey Dew 36.44% and Co-2 43.83%). The explant

of genotype Honey Dew produced 5 to 21 embryos per explant while 7 to 25 embryos was observed in explants of the cv. Co-2. Maximum globular embryos per explant were obtained in media with 3.0 mg/l 2,4,5-T and also in both cultivars. The genotype Co-2

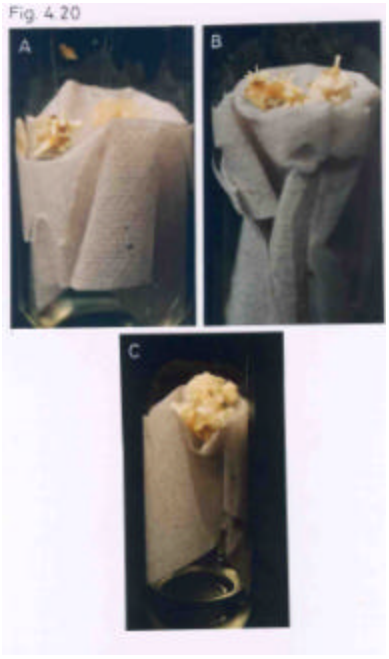


Fig. 4.20

A. Somatic embryos developing from the mature embryo explant of papaya in presence of 2.0 mg/l 2,4-D in liquid the MS basal liquid medium, cv. Co-2
B. Papaya somatic embryos developing from the mature embryo explant in presence of 3.0 mg/l 2,4-T in liquid MS liquid basal medium, cv. Honey Dew
C. Loose friable callus forming in the explant that did not responded producing somatic embryos

was better than Honey Dew in terms of both percentage induction of somatic embryogenesis and also number of globular embryos per explant.

From the above study, it can be observed that 3.0 mg/l 2,4,5-T responded best both in terms of percent induction and globular embryos per explant in Co-2 cv..

Therefore, this particular hormonal concentration and the Co-2 cv. were selected for further experimentation.

Induction of somatic embryogenesis in media containing auxin was reported earlier in papaya (Fitch 1990,1993; Castillo *et al*. 1998; Bhattacharya *et al*. 2002) and other crops (Sagare *et al*. 1993; Suhasini *et al*. 1996). Although 2,4-D is the most commonly used hormone, our earlier results with 2,4,5-T prompted us to test this hormone with mature embryo also. Earlier 2,4,5-T was used to induce somatic embryogenesis in other crops (Sagare *et al*. 1993; Suhasini *et al*. 1996).

4.3.3.2 Development of globular stage somatic embryos into cotyledonary stage

After 8 weeks of incubation, globular staged somatic embryos started appearing on the shoot pole region of the mature embryo explant (Fig. 4.21A). These embryos were

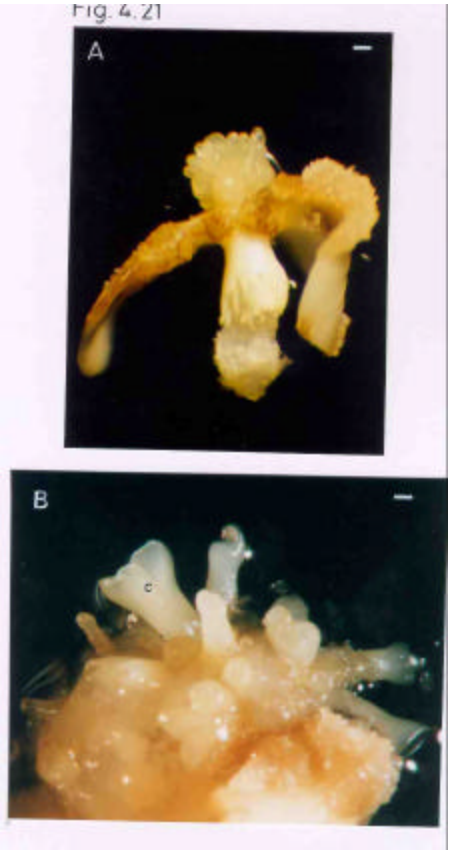


Fig. 4.21

A. Globular somatic embryos produced from shoot pole region of the mature embryo explant of papaya, cv. Co-2. Bar=263 µm

B. Cotyledonary structures developing from the mature papaya embryo axis. c- cotyledonary staged embryo, cv. Co-2. Bar=217 µm

loosely attached to the explants and were similar in their appearance like those obtained from immature embryos. Hundred and fifty globular embryos derived on liquid induction medium were subcultured on the same medium (liquid MS medium supplemented with 3.0 mg/l 2,4,5-T) in test tubes for their further development into cotyledonary stage somatic embryos (Fig. 4.21B). Thirty eight percent of the subcultured embryos developed into cotyledonary stage embryos. In another 4 weeks of incubation, globular embryos passed through torpedo and cotyledonary stage embryos similar to that of the stages observed in somatic embryogenesis from immature zygotic embryos. Scanning Electron Micrograph study and histological LS were carried out to reveal their origin (Fig. 4.22).

In general, it took about 12-13 weeks to obtain cotyledonary stage somatic embryos from the first day of inoculation. When a total of 500 embryos were cultured on liquid MS medium supplemented with 3.0 mg/l 2,4,5-T, 220 explants formed globular embryos. Again when 150 globular embryos were cultured, 57 cotyledonary stage embryos were obtained. Among them, 38% normal cotyledonary stage embryos was achieved. Remaining embryos showed abnormal morphology.

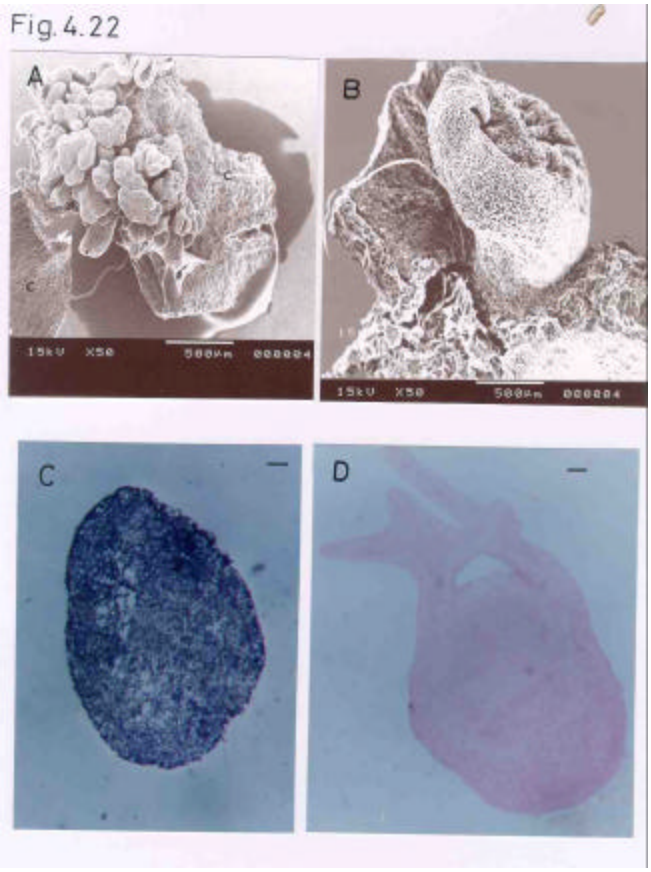


Fig. 4.22

A. Scanning electron micrograph of a mature embryo explant showing direct formation of somatic embryos from the shoot pole region, c-cotyleons

B. Scanning electron micrograph of a single cotyledonary shaped papaya somatic embryo obtained from mature embryo axes

C. Longitudinal section of a single papaya globular staged embryo obtained from mature embryo axes Bar=100µm

D. Longitudinal section of a single papaya cotyledonaty staged embryo obtained from mature embryo axes Bar=200 µm

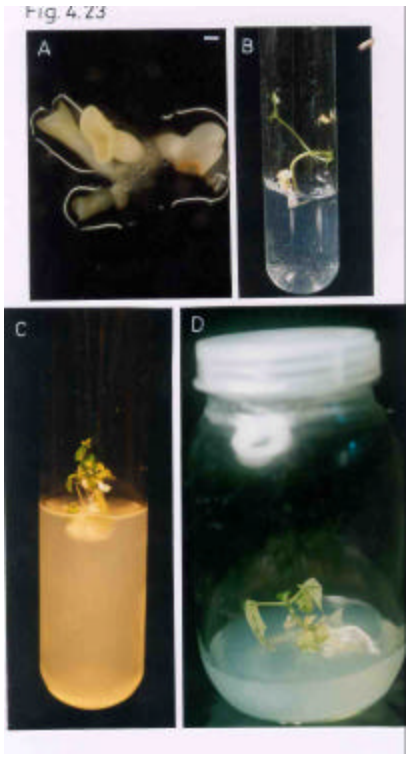


Fig. 4.23

A. Mature somatic embryo of papaya, derived from mature embryo axes explant, cv. Co-2. Bar=500 µm

B. Germination of papaya somatic embryos in MS basal medium supplemented with 1 mg/l GA_3 and solidified with 0.2% phytagel, cv. Co-2

C. Germination of papaya somatic embryos in MS basal medium supplemented with 1 mg/l GA_3 and solidified with 0.75% agar, cv. Co-2

D. Forty five days old normal somatic embryo germinated papaya seedling cv. Co-2

Abnormal embryos were characterized either by absence of shoot or root primordia or uneven growth of cotyledons or fused cotyledons. These abnormal embryos failed to develop further. The normal cotyledonary staged embryos were selected on the basis of presence of shoot and root primordia and even growth of cotyledons. Thus fifty-seven normal cotyledonary embryos were obtained. Similar morphological variations in somatic embryogenesis of other crops were reported (Hazra *et al.* 1989; Hartweek *et al.* 1988)

4.3.3.3 Maturation and conversion of somatic embryos

Maturation of the somatic embryos was done as described earlier with embryos obtained from immature embryo axes explant. Out of 57 cotyledonary embryos transferred, 32 embryos could be matured (Fig. 4.23 A), while others formed callus or turned necrotic. These mature embryos were again transferred to MS basal medium supplemented with 1mg/l GA_3, solidified with 0.75% agar or 0.2% phytagel for their germination (Fig. 4.23 B, C). Embryos incubated in growth regulator free MS medium produced only roots without any development of shoot apex (Fig. 4.24B). However, with GA_3 at a level of 1mg/l in the medium supported 25% conversion of the embryos. Thus out of 32 mature somatic embryos, 8 embryos get converted and formed normal plantlets (Fig. 4.23D). These plantlets were similar in appearance to the immature embryo derived plants.

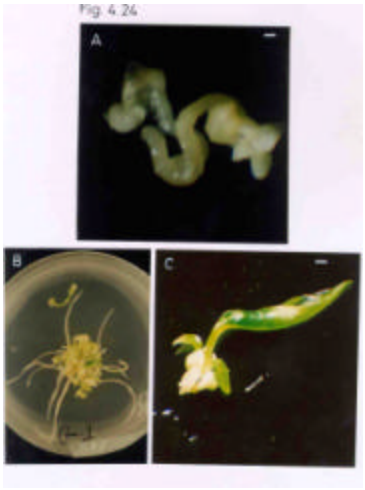


Fig. 4.24

A. Fused papaya somatic embryos joined at the base cv. Washington. Bar=166 µm
B. Root formation in the papaya somatic embryos (induced from mature explant) when cultured in MS basal medium without any growth regulator cv. Honey Dew
C. Abnormal growth of papaya somatic embryos (induced from mature explant) showing developing shoot apex without root formation cv. Honey Dew. Bar=270 µm

4.3.3.4 Transfer of plantlets to soil

On transfer of 8 plantlets to pots (8 cm) containing a potting mixture of sand, soil and farm yard manure (1:1:1 w/w) for 30 days, 25% of the plantlets survived. Thus 2 plants obtained were hardened and transferred to pot (Fig. 4.25).

4.4 Parameters studied in mature embryo axis

The following parameters were studied with the mature embryos of cv. Co-2 in presence of 3.0 mg/l 2,4,5-T in the somatic embryo induction medium.

4.4.1 Effect of basal medium

Basal media such as MS, B5, 1/2 MS, Whites, MBG were tested in presence of 3.0 mg/l 2,4,5-T and 3% sucrose. MBG media was found to be the best medium as it supported 62% induction and 28 embryos per explant, followed by MS and B5media (Table 4.17). White's media resulted least number of embryos while 1/2 MS failed to support induction of somatic embryogenesis in explants. Medium composition were reported to affect *in vitro* growth of papaya in earlier studies (Drew 1987; Medora *et al*.1984)

Fig. 4.25

A. Hardening of papaya somatic embryo derived seedlings in 8 cm pots

B and C. Two plants of cv. Co-2 obtained after hardening of mature somatic embryo derived seedlings

Table 4.17 Effect of basal medium on induction of somatic embryogenesis of papaya from mature embryo axes

Basal medium	% induction of embryos	Globular embryos per explant
MS	43.83±1.68 [b]	25.88±1.01 [a]
MBG	62.77±2.54 [a]	28.03±1.77 [a]
B5	29.11±2.00 [c]	16.74±2.20 [b]
WH	09.51±1.30 [d]	09.44±1.60 [c]
1/2MS	NR	NR

Values with same letter (a-d) in a column are not statistically different at $P = 0.05$

NR-Not responded

Although in majority of the cases MS basal medium is used, but in this experiment MBG medium showed best response which shows that mature papaya embryos need both high concentration of salts and vitamins for somatic embryo induction. As the MBG medium elicited best response, this medium was used for all the future experiments with mature embryo explant for somatic embryo induction.

4.4.2 Effect of pH

The effect of pH has not been critically evaluated although it has been shown that the capacity of cell cultures to utilize NH_4^+ as the sole N source depends on keeping the pH of the medium above 5.0 (Dougall & Verma 1978; Thorpe 1988). Medium pH has a pronounced effect on the growth of tissues *in vitro* and influences some plant developmental process (Oewn *et al.* 1991). The rates of heating and cooling, culture composition and sterilization temperature may affect post sterilization pH (Owen *et al.* 1991).

The effect of pH of the medium (5.5, 5.8, 6.0, 6.5, 7.0, 7.5 and 8.0) was evaluated on the induction of somatic embryos from mature embryo axis explant. Medium pH in the range 5.8-6.0 supported a good embryogenic response (62 and 55% response) (Table 4.18). Higher pH (7.0,7.5,8.0) of the medium was found to be inhibitory for

embryogenesis, similar to an earlier report in soybean (Lazzeri *et al*. 1987) and the explants showed profuse callusing.

Table 4.18 Effect of medium pH on induction of somatic embryogenesis of papaya from mature embryo axes

Medium pH	% induction of embryos	Globular embryos per explant
5.5	43.07±1.89 [c]	19.40±2.12 [c]
5.8	62.77±2.54 [a]	28.03±1.77 [a]
6.0	55.44±2.45 [b]	25.48±0.45 [b]
6.5	41.73±0.45 [c]	15.00±1.00 [d]
7.0	38.51±2.56 [cd]	12.37±2.46 [e]
7.5	22.48±1.83 [e]	08.40±0.61 [f]
8.0	11.70±1.46 [f]	02.16±0.76 [g]

Values with same letter (a-g) in a column are not statistically different at $P = 0.05$

4.4.3 Effect of temperature

Not much work was carried out on the effect of temperature treatments on somatic embryo induction although cold treatments have proved valuable in some cases (Rajasekaran *et al*. 1982; Tuleke & Mc Granahan 1985). Mature embryo axis were cultured on three temperature like 25±2 ^{0}C, 28 ^{0}C and in 30 ^{0}C. Explant incubated at 25±2 ^{0}C showed a better response in comparison to 28 ^{0} and 30 ^{0}C in terms of both percent induction and number of embryos per explant (Table 4.19).

Table 4.19 Effect of temperature on induction of somatic embryogenesis of papaya from mature embryo axes in cv. Co-2

Temperature (^{0}C)	% induction of embryos	Globular embryos per explant
25±2	62.77±2.54 [a]	28.03±1.77 [a]
27	42.18±2.02 [b]	13.37±2.02 [b]

30	16.55±1.50 [c]	04.06±1.10 [c]

Values with same letter (a-c) in a column are not statistically different at $P = 0.05$

4.4.5 Effect of Silver nitrate ($AgNO_3$)

$AgNO_3$ is an ethylene inhibitor in plants which is reported to help in somatic embryogenesis (Songstad *et al.* 1991; Roustan *et al.* 1990). $AgNO_3$ may also serve as stress agent inducing endogenous ABA accumulation (Hauser *et al.* 1992), Ag^+ being a metallic ion may also promote somatic embryo production via an increase in the endogenous ABA levels (Kong and Yeung 1994,1995). $AgNO_3$ was supplemented in MBG medium with 1.0, 3.0 and 5.0mg/l and was evaluated for somatic embryo induction after 4 weeks (Table 4.20). In our experiments, no improvement in the embryogenic response was observed. But on the contrary, the % explants responding to induction of somatic embryo decreased and the explants showed profuse callusing.

Table 4.20 Effect of $AgNO_3$ on induction of somatic embryogenesis of papaya from mature embryo axes of cv. Co-2

$AgNO_3$	% induction of embryos	Globular embryos per explant
0	62.77±2.54 [a]	28.03±1.77 [a]
1	30.11±1.16 [b]	16.37±1.58 [b]
3	26.70±2.14 [b]	13.45±1.37 [c]
5	20.07±1.11 [c]	03.5±1.32 [d]

Values with same letter (a-d) in a column are not statistically different at $P = 0.05$

4.4.6 Effect of physical state of medium (solid v/s liquid medium)

In an attempt to see the effect of physical state of the medium on the induction of somatic embryos from mature embryo axes, it was observed that both percent induction and number of globular embryos per explant was more in liquid medium than solid one (Table 4.21). Also the duration of somatic embryo induction in liquid medium was 8 weeks compared to 12 –13 weeks incubation in solid medium. The probable reason

advocated for this is that liquid medium facilitate better aeration and easy diffusion and flow of nutrients and growth regulators by capillary forces to the explants (Constabel and Shyluk 1994).

Table 4.21 Effect of physical state of medium on somatic embryo induction in papaya from mature embryo axes

Medium state	% induction of embryos	Globular embryos per explant
Liquid	62.77±2.54[a]	28.03±1.77 [a]
Solid	54.33±1.15 [b]	20.18±2.75 [b]

Values with same letter (a-b) in a column are not statistically different at $P = 0.05$

4.5 Conclusion

The present study demonstrates the embryogenic potential of immature and mature embryo axes of papaya. Efforts were directed to standardize the plant regeneration protocol from both these explants. Among all the phytohormones tested for induction of somatic embryogenesis, 2,4-D and 2,4,5-T has been found to influence the large number of somatic embryos in both immature and mature embryo axis explants. Other phytohormones like Picloram, Dicamba has also resulted somatic embryogenesis with varied degree of response. Among all the concentrations of 2,4-D tested, maximum explant's response and induction of globular embryos per immature embryo explant was observed when cultured on media supplemented with 2 mg/l of 2,4,-D. On an average, the explant's response was found to be 86% and 28 embryos/explant in cv. Honey Dew, 93% and 21 embryos / explant in Washington and 76% and 23 embryos / explant in cv. Co-2. However, maximum conversion of globular staged embryo to cotyledonary staged embryo was obtained when cultured on media supplemented with 2,4-D at a level of 5mg/l in both cv. Co-2 and Honey Dew. 2,4,5-T at a level of 3mg/l has supported the maximum explant response, in terms percent induction of embryos in all 3 cultivars (79% in Honey Dew, 71% in Co-2 and 76% in Washington). Also, this concentration (3 mg/l) of 2,4,5-T was noted to be the optimum for maximum number of globular embryos per explant (29,27 and 33 respectively in Honey dew, Co-2 and Washington) in this study. These embryos could convert to cotyledonary staged embryos in the same induction

medium. Maturation of these embryos was achieved in media supplemented with ABA (0.1mg/l). Embryos that originated from medium supplemented with 2,4-D, germinated maximum (70% in Honey Dew, 72% in Washington and 73% in Co-2) in presence of GA_3 (1.0mg/l). Hormone free MS basal medium has also supported the germination of embryos of cv. Honey Dew and Co-2, which were originated from medium supplemented with 2,4,5-T (3.0 mg/l). However, medium supplemented with GA_3 (1.0 mg/l) has favoured the germination of embryos of cv. Washington. Among the various parameters tested for improving the induction of somatic embryogenesis, MS as basal medium, sucrose (3%) as carbohydrate source, agar as gelling agent (0.75%) and culture of embryos in dark incubation has been found to exhibit a positive effect on increase in number of somatic embryos.

Advantages with mature embryo axes lies in the fact that seed material can be used through out the year without maintaining plants in green house or field plantings, while with immature embryo axes, the response is comparatively more and also the chances of contamination are less as the immature seeds are already sterile. Presence of 3.0 mg/l 2,4,5-T in the induction medium has resulted in maximum response of explants in terms of somatic embryogenesis in Honey Dew (36.44%) and in culitvar Co-2 (43.83%). These embryos were matured in medium containing ABA (0.1mg/l) and were germinated in presence of MS basal medium supplemented with GA_3 (1.0 mg/l). Among the various basal medium tested for somatic embryo induction in this study, MBG basal medium was found to be the best induction medium as it resulted 62.77% response and 28.03 embryos per explant in cv. Co-2. Culture of explants in liquid condition with filter paper bridges has exhibited the significant improvement in both explant's response and in number of somatic embryos in our study. Incubation of expants at a temperature of $25\pm2\ ^0$ C has also shown a positive influence in induction of somatic embryogenesis. Silver nitrate at all three levels tested did not exhibit a positive influence in somatic embryo induction in this study.

The somatic embryogenesis system developed here can be used for transformation experiments and also for synthetic seed technology as direct origin of embryos will lead to the production of genetically uniform plants (Gill & Saxena 1992).

Part of this chapter has been communicated as papers entitled "Somatic embryogenesis and plantlet regeneration in papaya cv. Honey Dew and Co-2" in Indian Journal Of Experimental Biology by Bhattcaharya J., Khuspe SS, Renukdas N and Rawal SK (2002) 40: 624-627. and also as

"2,4,5-T induced somatic embryogenesis in papaya" in Journal Of Applied Horticulture by Bhattacharya J and Khuspe SS (revision accepted 2002)

CHAPTER 5

IN VITRO INDUCTION OF MULTIPLE SHOOTS AND PLANT REGENERATION FROM EMBRYO AXIS AND SHOOT TIP EXPLANT OF PAPAYA

5.1 Introduction

In vitro plant regeneration or micro propagation through tissue culture is an important step in the success of any crop improvement program. A rapid, simple and efficient plant regeneration protocol is also a major prerequisite for genetic transformation of crop plants.

Propagation under *in vitro* culture conditions can be achieved by using different explants such as axillary bud, shoot apex, hypocotyl, leaf and root etc. Plants can be propagated by tissue culture methods in three different ways: 1) by inducing the pre-existing shoot primordia to grow and multiply 2) by shoot morphogenesis either directly from the explant or from unorganized tissues and by 3) somatic embryogenesis (direct or indirect). The commitment of competent cells for morphogeneis is affected by many factors including complex interactions between genotypes, the explant (its stage of development) and culture medium etc. Morphogenesis is triggered usually after competent cells are subcultured into a less complex medium allowing the expression of new developmental potential (Thorpe 1983). The changes in the macro, micro nutrient ratio and addition of various substances such as charcoal, organic compounds (vitamins, amino acids polyamines etc), carbohydrates, light intensity, pH, water potential, temperature, gaseous atmosphere and container shape etc. were found to affect the plant morphogenesis (Tran Thanh Van 1981). It is believed that only a single factor cannot affect the morphogenesis but it is the combination of various stimulating factors, which needs to be applied to the cells not only in right amount but also on right sequence and under right culture conditions (Stewards *et al*. 1964) for morphogenesis.

The process of *in vitro* plant propagation mainly consist of three steps: i) induction of shoot buds and their multiplication ii) elongation of shoot buds into shoots and iii) *in vitro* and *ex vitro* rooting of shoots to form plantlets. The process has several advantages like, propagation is simple, rapid and plants obtained are true to type, cultures can be initiated from very small segments of the mother plant, propagation in most of the species is possible through out the year, greater degree of control over chemical, physicaland environmental factors, possibilities of rejuvination from the mature tissues (Ahuja 1986).

5.1.1 *In vitro* plant regeneration in papaya

In vitro plant regeneration in papaya has been achieved by somatic embryogenesis and through organogenesis. Although somatic embryogenesis has been reported in exotic cultivars of papaya (Litz and Conover 1981,1982; Yamamoto and Tabata 1989; Fitch 1990, 1993), however, reports on Indian cultivars till date is meager. Details of these have already been discussed in the chapter 1.

Plant regeneration via organogenesis is also reported in papaya. Reports on papaya regeneration via organogenesis have been mentioned in chapter 1. Early reports (Mehdi and Hogan 1976; Yie and Liaw 1977) failed to achieve shoot proliferation in papaya. Litz and Conover (1977, 1978a) developed a procedure of shoot proliferation from shoot tips of field grown papaya. Regeneration of plants from callus cultures was obtained from stem segments of Indian cultivars of papaya (Arora and Singh 1978b). Shoot tip and shoot bud (axillary and lateral bud) explants have also been used for plant regeneration study in papaya (Rajeevan and Pandey 1983; Drew and Smith 1986; Winner 1988; Reuveni et al.1990; Mondal et al. 1990; Lai et al. 1998). Yang and Ye (1992) and Hossain et al. (1993) reported plant regeneration from petiole explants of papaya. However, shoot regeneration from embryo axis of papaya has not been reported earlier. The embryo axis explant has many advantages as shoot regeneration is direct, and is not prone to somaclonal variation and chromosomal abnormalities (Saeed et al. 1997).

With a view to develop a suitable regeneration protocol for Indian cultivars, the potential of embryo axis for shoot multiplication were tested *in vitro*. Also, the use of shoot tips for clonal propagation was studied in two popular cultivars of India.

The present chapter deals with the following two aspects:

1. Establishment of a plant regeneration protocol from embryo axis explant.
2. Studies on plant regeneration protocol from shoot tip explants.

5.2 Materials and methods

5.2.1 Plant material

Seeds of papaya cultivars Honey Dew, Washington and Co-2 were surface sterilized as described in chapter 3. Immature fruits (90-110 days old) were also surface sterilized following the same procedure.

5.2.2 *In vitro* induction of multiple shoots

Experiments were carried out to study *in vitro* induction of multiple shoots in embryo axes and shoot tip explants of papaya. Three cultivarss Honey Dew, Washington and Co-2 were used for the embryo axis experiments and for shoot tip multiplication only Honey Dew and Co-2 were tested. Material and methods has been divided into two subsections: A and B.

5.2.2.1 A : Explant preparation- immature embryo

Immature embryos of three cultivars were isolated aseptically from the fruits (90-110 days old) in a process described earlier (Chapter2). Two types of explants were prepared from immature embryos. One is the full embryo with radicle and plumule and other is the cotyledons (Fig.5.1A) devoid of embryo axis. Cotyledons were cut at the base of joint where they are attached to the embryo.

5.2.2.2 Effect of basal media on sprouting response of embryo axis

In a preliminary set of experiment, influence of two basal media, MS basal media (Murashige and Skoog 1962) and MS salts + B5 vitamins + 2 mg/l glycine (herein after this media combination will be termed as MBG) on sprouting response of 100 embryos of all the three cultivars were tested.

5.2.3 *In vitro* induction of multiple shoot from immature embryo

The selected basal medium (MBG) was then supplemented with various phytohormones to determine the morphogenetic response. The most responsive phytohormones thereafter, were evaluated for induction of multiple shoots from immature embryo axis and cotyledon explant of three cultivars. The media were supplemented with 2% sucrose and 0.75 agar (Hi-media, India) and the pH was adjusted to 5.8 before autoclaving. The cultures were incubated at 25±2 ^{0}C under cool white fluorescent light at 27 μEm^{-2} S^{-1} with a 16 h photoperiod.

5.2.3.1 Effect of various phytohormone on morphogenetic response

All the explants of 3 cultivars were cultured in petridishes (85mm x 15mm) containing MBG medium supplemented with various phytohormones such as BAP, Kin, Zeatin, TDZ and NAA (0.5-5.0 mg/l) either alone or with a combination of BAP (0.05-2.0 mg/l) and NAA (0.1 and 0.5 mg/l). Hundred explants per treatment in a replication of 3 sets were tested for this experiment. After 3 weeks of incubation, these were again

transferred to fresh medium and were incubated for further 3 weeks. At the end of 6th week the nature of explants response was recorded.

5.2.3.2 Effect of various basal media on multiple shoot induction

Other basal media were tested with the selected phytohormones to evaluate the shoot induction effect. These are MS basal medium, B5 medium, White's medium, 1/2 strength MS and modified MS salt +B5 vitamins +2mg/l glycine (MBG). All these basal media were supplemented with either TDZ or BAP and NAA combination. In each case 100 embryos were cultured. At the end of 6th week, the response of the explants was observed.

5.2.3.3 Effect of carbon sources on multiple shoot induction

Influence of various source of carbon was evaluated on their efficiency in inducing multiple shoots. The explant used was the immature embryos. These were cultured on MBG medium supplemented with TDZ (0.5 mg/l) and also in BAP and NAA combination (0.5: 0.1 mg/l). The culture and experimental conditions were kept same as mentioned above.

5.2.2.2 B: Explant preparation- shoot tip

The seeds were germinated in MS basal medium as described in chapter 3. Shoot tip explants were prepared from 20 day old seedlings. Shoot tip was cut from the top measuring 1-2 cm. Only 2-3 new leaves were retained and cultured (Fig.5.1B).

5.2.4 *In vitro* induction of multiple shoot from shoot tip

The influence of various culture conditions i.e phyto hormones, culture vessels, age of seedling, physical state of the medium were tested on induction of multiple shoots from shoo tip. MBG formulations were used as basal medium for the whole experiment. All the media were supplemented with 3% sucrose and 0.75 % agar. The pH of the medium was adjusted to 5.8 before autoclaving. All cultures were incubated at 25±2 ^{0}C under cool white fluorescent light at 27 μEm^{-2} S^{-1} with a 16 h photoperiod.

5.2.4.1 Effect of phytohormones on morphogenetic response

In a preliminary experiment different phytohormones were tested to assess the morphogenetic response of the plant. Shoot tips were excised from 20 day-old seedling and were cultured in test tubes containing MBG medium supplemented with BAP, NAA, Kinetin, 2 iP (0.1-2mg/l) and NAA (0.1-0.5mg/l) singly or with combinations of BAP,

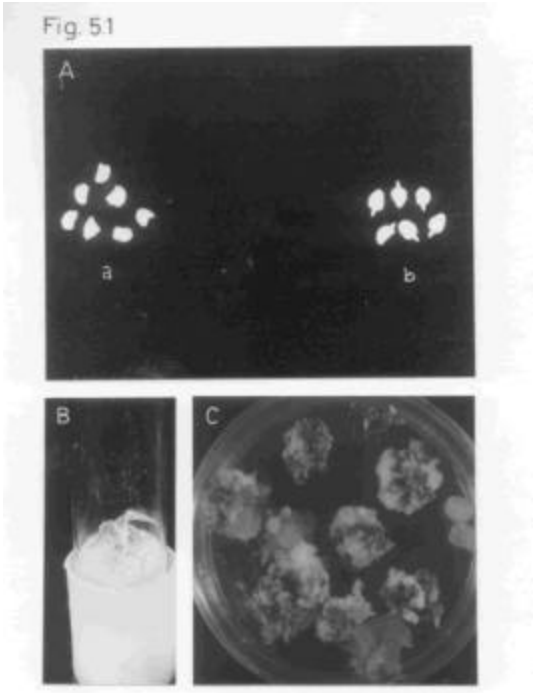


Fig. 5.1

A. a- immature cotyledons b-Immature embryos

B. Shoot tip explant used for shoot multiplication

C. Multiple shoot formation from immature embryo axis of papaya

Kin and 2iP (0.1-2mg/l) each with NAA (0.1 and 0.5mg/l). Cultures were incubated for 5-6 weeks under conditions mentioned above. Thirty explants were cultured per treatment. After 6 weeks the nature of response was observed.

5.2.4.2 Effect of seedling age on induction of multiple shoot

In this experiment, shoot tips were excised from 7-, 20- and 30 day-old *in vitro* raised seedlings. These explants were cultured on MBG medium supplemented with the best BAP and NAA combination (0.5+0.1 mg/l) as observed in the study of growth regulators above. The cultures were incubated for 5-6 weeks under conditions mentioned above. Thirty explants were cultured per treatment. After 6 weeks only those explants which formed a minimum of one shoot on an average were considered as responded (expressed as % explant response), while formation of more than two shoots by an explant was considered as induction of multiple shoots.

5.2.4.3 Effect of culture vessel on sprouting response

In a separate experiment, the efficiency of multiple shoot formation was evaluated from 30-day-old shoot tip explant when cultured in test tubes and glass bottles. These explants (30 explants per treatment) were cultured on MBG medium supplemented with BAP and NAA combination (0.5+0.1 mg/l). Cultures were incubated as above.

5.2.4.4 Effect of physical state of the medium used

Effect of the physical state of the medium was also tested in inducing multiple shoots from the shoot tip explant. For this, MBG medium supplemented with BAP and NAA combination (0.5+0.1 mg/l) was either solidified with agar (0.75%) or kept in liquid form (without adding gelling agent). Explants cultured on liquid medium were incubated in the rotary shaker with 100 rpm under 16 h photoperiod condition at 25± 2 ^{0}C. Thirty explants were cultured per treatment.

5.2.5 Elongation of shoots obtained from immature embryo

Multiple shoot bunches obtained from embryo explants were transferred to hormone free MBG basal medium containing 0.75% agar and 3% sucrose or to media supplemented with various combination of GA_3 (1.0-5.0 mg/l). The cultures were incubated under the conditions mentioned as above for 15 days. Twenty shoot bunches were treated in each treatment.

5.2.5.1 Elongation of shoots obtained from shoot tip

For elongation, twenty shoots were separated from proliferated shoot tips and were transferred to phytohormone free basal medium in culture tubes.

5.2.6 Rooting and Hardening

The elongated shoots (3-5 cm) were excised and transferred to half strength MBG medium containing (1-4 mg/l) of IBA for rooting. Ten shoots per treatment were tested and the experiment was repeated three times. The cultures were incubated as above for 3 weeks. The rooted plantlets were hardened in pots with sand: soil: vermiculite (1:1:1) mixture at 25±2 ^{0}C under diffuse light (16/8 h photoperiod) condition for 2-3 weeks.

5.2.7 Statistical analysis

The data were analyzed by ANOVA technique for a Completely Randomized Design and the treatment means were compared.

5.2.8 Histology and SEM studies

To confirm the origin of shoots the embryo axis with multiple shoots were fixed in formalin: acetic acid: alcohol (5:5:90 vol/vol) for 48 h. Histology was carried out as described in the chapter 2 (Materials and Methods). A scanning electron microscope study of the explants with shoot buds and developing shoots were also carried out.

5.3 Results and Discussion

5.3.1 Induction of multiple shoots from immature embryo

Similar to the material and methods section, the results of plant regeneration from all the explants have been described in two different sections. The following section describes the results obtained from embryo axis and cotyledon explant of papaya.

In an initial study to find out a suitable basal medium for multiple shoot induction, we tested MS and MBG on initial sprouting response from immature papaya embryo. From the experiment (Table 5.1), MBG basal medium was found to exhibit 90% sprouting response in both Honey Dew and Co-2 and 95% in Washington. However, explants of these cultivars in presence of MS medium resulted only 64, 65 and 56 % response in Honey Dew, Co-2 and in Washington respectively. Therefore, MBG medium was selected for our further experiments with both immature embryos and cotyledons.

Table 5.1 Effect of two basal media on initial sprouting response of papaya from immature embryos

Basal media	Cultivar		
	Honey Dew	Co-2	Washington
MS	64.33±6.02 [b]	65.66±4.04 [b]	56.00±5.29 [b]
MBG	90.00±2.00 [a]	90.00±3.60 [a]	95.66±4.04 [a]

Values with same letter (a-b) in a column are not statistically different at P = 0.05. HD- cv. Honey Dew. Co-2 cv. Co-2 and WA- cv. Washington

5.3.1.1 Effect of phytohormones on morhphogenetic response from immature embryo axes and cotyledons of papaya.

In an initial experiment, immature embryo explants and cotyledons of papaya when cultured on MBG medium with BAP, Kin, NAA and TDZ at various concentrations resulted in different degree of response depending on the phytohormone tested (Table 5.2). Varied effect of growth regulator was also studied earlier in papaya tissue *in vitro* (Falcone and Leva 1986; Burikam *et al*. 1988; Islam *et al*. 1993). Media supplemented with BAP, Kin, Zeatin and NAA (0.1-5.0mg/l) alone also did not produce or support any multiple shoot development. The explants developed only callus or single shoot with callus at the base. However, TDZ alone and a combination of BAP and NAA at all concentrations tested produced shoot buds (Table 5.2).

To evaluate the effects of TDZ and BAP + NAA combination separately, the immature embryo and cotyledons were also cultured separately in presence of both the hormones. The results obtained are discussed below.

Table 5.2 Effect of phytohormones on morphogenetic response in immature embryo and cotyledon explant of papaya.

Phytohormone	Conc. (mg/l)	Nature of response	
		Embryo	Cotyledon
BAP	0.1	SSC	NR
	0.5	SSC	NR
	1.0	C	NR
	2.0	C	NR
	5.0	NR	NR
NAA	0.1	C	NR
	0.5	C	NR
	1.0	C	NR
	2.0	C	NR
	5.0	NR	NR
BAP+ NAA	0.05+0.1	SB	S
BAP+ NAA	0.05+0.5	SB	S
BAP+ NAA	0.1+0.1	SB	S
BAP+ NAA	0.1+0.5	SB	S
BAP+ NAA	0.5+0.1	SB	S
BAP+ NAA	0.5+0.5	SB	S
BAP+ NAA	1.0+0.1	SB	S
BAP+ NAA	1.0+0.5	SB	S
BAP+ NAA	2.0+0.1	SB	S
BAP+ NAA	2.0+0.5	SB	S
Kin	0.1	SSC	NR
	0.5	SSC	NR
	1.0	C	NR
	2.0	C	NR
	5.0	C	NR

TDZ	0.1	SB	SB
	0.2	SB	SB
	0.5	SB	SB
	1.0	SB	SB
	1.5	SB	SB
	2.0	SB	SB
	5.0	SB	SB
Zeatin	0.1	C	NR
	0.5	C	NR
	1.0	C	NR
	2.0	C	NR
	5.0	C	NR

C: callus: NR: not responded: SSC: single shoot with callus at the root pole: SB: shoot bud. S: Swelling

5.3.1.1.1 Effect of TDZ on shoot induction from embryo axis and cotyledon explants

The immature embryo explant responded very quickly to the TDZ containing media. It became thick and turned green in colour. Both immature zygotic embryo axes and immature cotyledons when cultured on MBG basal media containing 3% sucrose and supplemented with thidiazuron (TDZ 0.5-5.0 mg/l) responded within 6-15 days of incubation. In all instances, the explants showed visible swelling and callus formation. Whitish yellowish callus was induced from the root pole of the embryo axis after 6-7 days. The meristematic region of the embryo axis expanded rapidly without any callus formation (Fig.5.2A), followed by proliferation of large number of shoot initials (Fig.5.2B). The number of explant showing shoot initiation ranges from 7 to 95% in Honey Dew, 7 to 91% in Co-2 and 11 to 94% in Washington (Table 5.3) during the second week of culture depending on the concentration of TDZ added in the media. These shoot initials, later differentiated into individual shoots with shoot tips after four weeks of incubation (Fig. 5.1C). Scanning electron micrograph observations showed the direct formation of shoot buds with developing shoots from the embryo axis explant (Fig.5.16A). The histology of the embryo axis after 15 days of development also

confirms the direct formation of shoot buds from embryo axis (Fig.5.17A). A L.S (longitudinal section) of expanded explants after 3 weeks showed the formation of meristematic regions around shoot pole region in a circular way. Each of these meristematic pockets later on gave rise to single shoot buds with well developed meristems (Fig. 5.18B). Embryos when cultured on medium without any phytohormone resulted in occasional germination or remained quincascent. Immature cotyledons cultured in the same medium also expanded rapidly, twice of their original size and developed callus at the cut ends after 6-7 days of incubation.

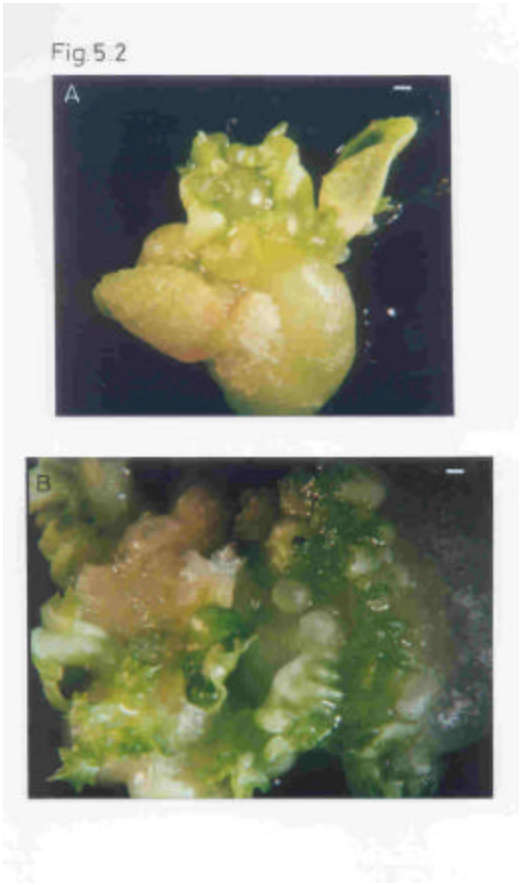


Fig. 5.2

A. Shoot initiation in embryo axis explant cultured on MBG medium containing TDZ 0.5 mg/l. Bar=318 µm

B. Development of shoot initials on the immature embryo axis after two weeks of incubation in presence of TDZ (0.5mg/l) in the MBG basal medium. Bar=269 µm

In case of embryo axis, media supplemented with TDZ at 0.5 mg/l supported on an average 14.62±1.88, 14.51±2.06 and 14.57±1.90 number of shoots in Honey Dew, Co-2 and Washington respectively (Fig.5.3B) from embryo axis. However, immature embryo axes cultured on media supplemented with TDZ at 2.0 mg/l resulted an average of 3.00±0.47 number of shoots in Honey Dew, 2.83±0.47 in Co-2 and 2.83±0.60 in Washington (Table 5.3). Rosette structures with leafy shoots were also observed when higher concentration of TDZ (5.0 mg/l) was used in the media.

Table 5.3 Effect of TDZ on multiple shoot induction from immature embryo axis of papaya.

Conc. Used (mg/l)	% Explant forming multiple shoot			No. of shoots per explant		
TDZ	**HD**	**Co-2**	**WA**	**HD**	**Co-2**	**WA**
0.0	G	G	G	G	G	G
0.1	62.20±0.58 [d]	60.00±1.00 [c]	63.33±1.00 [b]	4.22±1.35 [d]	04.66±1.50 [c]	04.53±1.50 [d]
0.2	86.66±1.00 [b]	80.00±1.00 [b]	85.53±1.52 [a]	05.59±1.57 [c]	05.55±1.58 [c]	05.84±1.73 [c]
0.5	95.53±1.15 [a]	91.10±0.58 [a]	94.43±1.52 [a]	14.62±1.88 [a]	14.51±2.06 [a]	14.57±1.90 [a]
1.0	71.10±1.15 [c]	66.66±2.00 [c]	67.76±2.88 [b]	07.64±1.54 [b]	07.91±1.68 [b]	07.84±1.67 [b]
1.5	44.43±0.58 [e]	46.66±1.73 [d]	47.76±1.52 [c]	04.89±1.80 [d]	05.11±2.12 [c]	05.14±2.01 [d]
2.0	44.43±1.52 [e]	48.86±1.53 [d]	46.66±1.00 [c]	03.00±0.47 [e]	2.83±0.47 [d]	02.83±0.60 [e]
5.0	07.76±1.15 [f]	07.76±0.58 [e]	11.00±1.52 [d]	*	*	*

*could not be counted. MS medium without TDZ served as control. Values with same letter (a-f) in a column are not statistically different at P = 0.05. HD- cv. Honey Dew. Co-2- cv. Co-2 and WA- cv. Washington

Similarly, the development of shoot meristems were also observed on the cultured cotyledons after the second week of incubation (Fig.5.3A). Depending of the TDZ concentrations added in the media, response of the explants varied with a range from 6-94% in Honey Dew, 5 to 88 % in Co-2 and 5 to 92% in Washington cultivars (Table 5.4). These meristems later developed into shoots after 5-6 weeks of incubation. Figure 5.16B shows a single shoot bud arising from cotyledon explant. Among the various TDZ

concentrations (0.1-5.0 mg/l) tested, 0.5mg/l was found to be the best treatment in terms of shoot induction from both the types of explant used.

Similar to embryo axis, the cotyledons also produced rosette structures in media supplemented with 2 and 5 mg/l of TDZ. However, explant developed maximum number of shoots in all three cultivars i.e Honey Dew, Co-2 and Washington (14.22±2.0, 14.26±1.95 and 14.42±1.92 respectively) when cultured on medium added with 0.5 mg/l TDZ (Table 5.4). No response was observed in explants cultured on control medium. In all the cases, shoots arose directly from the explant without any intermediary callus growth. TDZ was observed to elicit a typical concentration dependent response from the cultured explants. None of the cultured embryo axis explants and cotyledons developed multiple shoots in absence of TDZ. It was observed that number of shoots per explant increased with TDZ concentration up to 0.5 mg/l. TDZ concentrations above 0.5 mg/l in the medium resulted in a decline in the percentage of embryo axis explants forming multiple shoots. The number of shoots developed from each explant was also found to be concentration dependent. Maximum number of shoots (14 shoots per explant) (Table 5.3 and 5.4) was regenerated in presence of 0.5 mg/l TDZ, in both embryo axis and cotyledonary explant in all three cultivars tested. Explants regenerated less number of shoots on media supplemented with either lower or higher than 0.5 mg/l of TDZ concentrations. Progressive condensation and decrease in shoot length was also observed with TDZ concentration higher than 0.5 mg/l in the medium. Only leafy shoots without any shoot tips were formed in media with 2.0 and 5.0 mg/l TDZ in case of immature cotyledons.

Table 5.4. Effect of TDZ on multiple shoot induction from immature cotyledons of papaya.

Conc. Used (mg/l)	% Explant forming multiple shoot			No. of shoots per explant		
TDZ	**HD**	**Co-2**	**WA**	**HD**	**Co-2**	**WA**
0.0	NR	NR	NR	NR	NR	NR
0.1	61.10±0.58 ^{d}	58.86±1.53 ^{d}	60.00±1.00 ^{d}	04.30±1.38 ^{cd}	03.90±1.10 ^{cd}	04.11±1.25 ^{cd}
0.2	85.53±1.15 ^{b}	81.10±1.15 ^{b}	83.33±1.00 ^{b}	05.62±1.56 ^{c}	05.40±1.58 ^{c}	05.53±1.61 ^{c}
0.5	94.43±1.53 ^{a}	88.86±1.53 ^{a}	92.20±0.58 ^{a}	14.22±2.00 ^{a}	14.26±1.95 ^{a}	14.42±1.92 ^{a}

1.0	72.20±1.52 [c]	67.76±0.58 [c]	70.00±1.73 [c]	07.66±1.51 [b]	07.30±1.55 [ab]	07.50±1.60 [b]
1.5	43.33±1.00 [e]	40.00±1.73 [e]	42.20±0.58 [e]	04.91±1.81 [cd]	04.41±1.47 [cd]	04.72±1.64 [cd]
2.0	43.33±1.00 [e]	38.86±0.58 [e]	41.10±1.53 [e]	*	*	*
5.0	06.66±1.00 [f]	05.53±0.58 [f]	05.53±0.53 [f]	*	*	*

*could not be counted. NR-Not responded. MS medium without TDZ served as control. Values with same letter (a-f) in a column are not statistically different at P = 0.05.HD- cv. Honey Dew. Co-2- cv. Co-2 and WA- cv. Washington

This has also been observed in immature embryo axis cultured on media supplemented with 5.0 mg/l TDZ. From Table 5.3 and 5.4 it can be concluded that embryo axis responded better in terms of number of shoots per explant than immature cotyledons. Therefore, all the further experiments were carried out with immature embryo axis. Similar observations were also reported earlier (Ozcan *et al*. 1996). Application of TDZ on papaya tissue culture has not been reported so far. Recently TDZ is being used in shoot regeneration experiments of various crops (Kim *et al*. 1997; Magioli *et al*.1998; Sujatha and Reddy 1998). TDZ is one of the several substituted ureas such as N-N' diphenyl urea (Mok *et al*. 1980) and N-(2-chloro-4-pyridyl)-N' phenyl urea (Fellman *et al*.1987) that have been investigated for cytokinin activity (Huetteman and Preece 1993; Lu 1993). It is also reported that lower concentration of TDZ can stimulate axillary shoot proliferation in many woody plants (van Nieuwkerk *et al*. 1986; Chalupa 1988;) whereas TDZ at higher concentration causes a reduction in shoot formation (Magioli *et al*. 1998; Goldfarb *et al*. 1991; Leblay *et al*. 1991). Our experiments with TDZ in plant regeneration in papaya also confirm the findings of the above experiments.

5.3.1.2 Effect of BAP and NAA combination on induction of shoot buds from immature embryo

Although in medium supplemented with BAP and NAA, the immature embryo explant showed visible signs of swelling within 1 week of culture initiation, the response was only from the shoot apex region of the embryo axis. Unlike TDZ, the cotyledonary part did not form multiple shoots. Explants only thickened, increased in size and turned green in colour.It was observed that embryos when cultured on multiplication medium,

the meristematic region expanded greatly with white friable callus formation at the base, followed by proliferation of a large number of shoot buds.

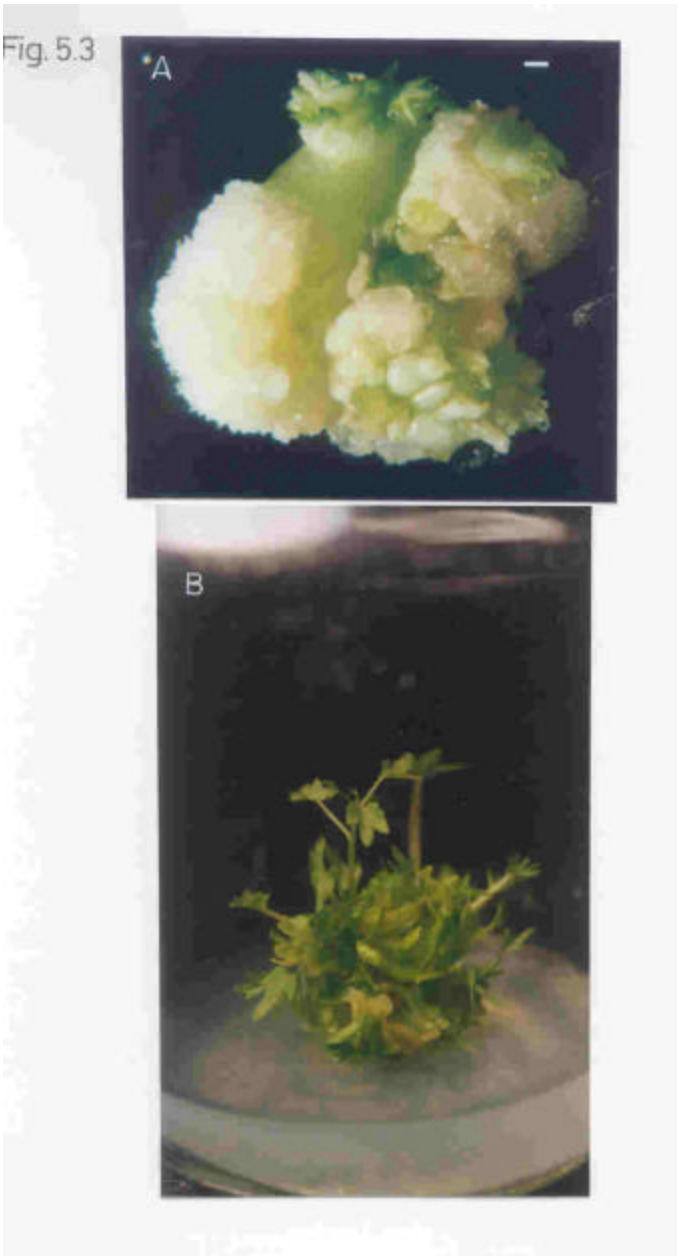


Fig. 5.3

A. Development of shoot initials on the immature cotyledons after two weeks of incubation in presence of TDZ (0.5mg/l) in the MBG basal medium. Bar=253 µm

B. Multiple shoots arising from immature embryo axis of papaya in presence of TDZ (0.5mg/l) in the MBG basal medium

Green shoot initials were noted on the callus formed on the explant after two weeks of inoculation (Fig.5.4A). The explant expanded greatly with white loose callus formation at base (Fig.5.4B).

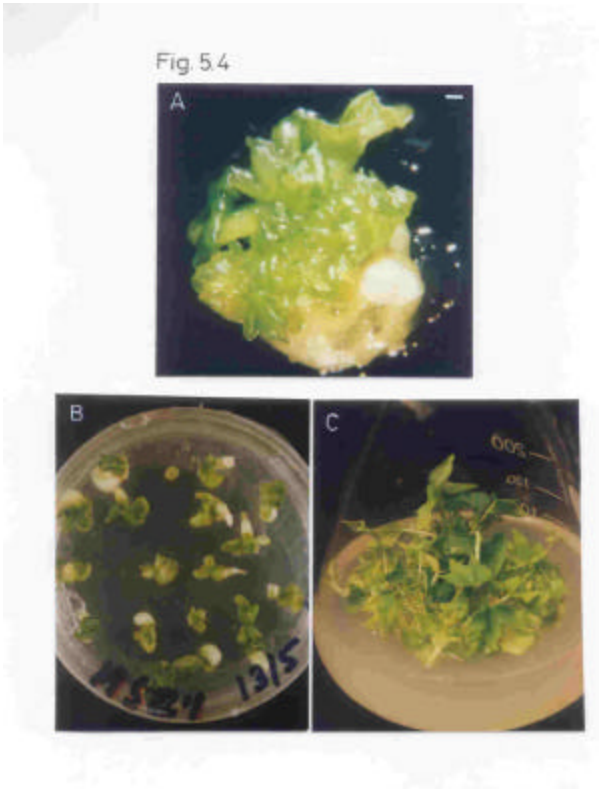


Fig. 5.4

A. Development of shoot initials on the immature embryo axis after two weeks of incubation in presence of BAP and NAA (1.00 and0.1 mg/l) in the MBG basal medium. Bar=233 µm
B. Formation of multiple shoots from immature embryo axis after three weeks of incubation in presence of BAP and NAA (1.00 and 0.1 mg/l) in the MBG basal medium
C. Growth of multiple shoots from immature embryo axis after forty five days of incubation in presence of BAP and NAA (1.00 and 0.1 mg/l) in the MBG basal medium

These shoot initials later developed into shoots. Highest number of shoots (14.27 shoots in Honey Dew, 14.25 shoots in Co-2 and 14.38 shoots in Washington) (Table 5.5) per explant was produced by BAP + NAA combination (1+0.1 mg/l) as observed after 45 days of culture initiation (Fig.5.4C). Explants cultured on media containing higher concentration of BAP developed less number of shoots. This may be because of large shoot developed by the pre existing meristems which inhibited adventitious shoot regeneration. Although the hormonal combination of BAP and NAA has been widely used in papaya micro propagation studies (Mondal *et al.*1990; Islam *et al.*1993), its effect on embryo axis explant has not been reported. The regenerated shoot mass were shifted to the basal medium without any phyto-hormone for further elongation.

Table 5.5 Effect of BAP and NAA on induction of shoots from papaya embryos

Conc. used (mg/l)	% explant forming multiple shoots			No. of shoots per explant		
BAP:NAA	**HD**		**WA**	**HD**	**Co-2**	**WA**
00.00:0.00	G	G	G	G	G	G
00.05:0.10	88.86±1.52 [f]	87.77±1.52 [d]	87.76±2.08 [bc]	02.77 ± 0.38 [g]	02.88±0.38 [i]	03.05±0.95 [g]
00.05:0.50	91.10±1.52 [d]	92.20±1.15 [b]	88.86±2.08 [b]	03.43± 0.80 [f]	03.37±0.80 [g]	03.54±0.66 [g]
00.10:0.10	90.00±1.00 [e]	91.10±0.58 [c]	88.86±1.52 [b]	04.40 ± 1.36 [f]	04.17±1.36 [f]	04.22±1.26 [f]
00.10:0.50	95.53±0.58 [a]	94.43±0.58 [a]	93.33±1.00 [a]	06.22±1.71 [e]	06.01±1.71 [e]	06.16±1.73 [e]
00.50:0.10	90.00±1.00 [e]	91.10±0.58 [c]	88.66±1.52 [b]	07.26±1.53 [d]	07.09±1.53 [d]	07.07±1.63 [d]
00.5:0.50	92.20±2.51 [c]	94.43±1.52 [a]	91.10±3.05 [b]	08.80 ±2.54 [c]	08.83±2.54 [c]	08.91±2.42 [c]
1.00:0.10	93.33±2.64 [b]	95.53±1.52 [a]	94.43±2.08 [a]	14.27±3.93 [a]	14.25±3.90 [a]	14.38±3.81 [a]
1.00:0.50	93.33±2.00 [b]	92.20±1.52 [b]	94.43±1.82 [a]	10.11±2.79 [b]	09.93±2.79 [b]	09.96±2.89 [b]
2.00:0.10	91.10±1.15 [d]	92.20±0.58 [b]	90.00±1.00 [b]	04.03±1.42 [f]	03.46±1.41 [g]	04.22±1.11 [f]
2.00:0.50	92.20±1.15 [c]	93.33±1.0 [a]	90.00±1.00 [b]	03.42±1.15 [f]	03.00±0.00 [h]	01.83±0.28 [h]

MS medium without BAP and NAA served as control. Values with same letter (a-i) in a column are not statistically different at P = 0.05.HD- cv. Honey Dew. Co-2- cv. Co-2 and WA- cv. Washington

5.3.1.2 Effect of basal media on multiple shoot formation from embryo axis

Response of the explants and shoot proliferation varies with the composition of the basal medium (Shekhawat *et al*. 1993; Das *et al*. 1996). Although two basal media were initially tested to determine the sprouting response of the explants, other basal media like B5, White's and 1/2 MS, were also experimented with the selected phytohormones 0.5 mg/l TDZ and BAP: NAA (1:0.1 mg/l) separately for multiple shoot regeneration from immature embryo axis. MBG medium was found to be the best basal medium in terms of the percent explant response with more than 96% in all the cultivars tested. The embryo axis responded least when inoculated in ½ MS basal medium. Highest number of multiple shoots (14.51±2.06 in Co-2, 14.62±1.88 in Honey Dew, 14.57±1.90 in Washington) was also achieved with MBG basal medium containing TDZ 0.5mg/l (Table 5.6). Except in the variety Honey Dew, the B5 basal media found to be the second most responsive basal medium for multiple shoot induction in this case.

Table 5.6 Effect of basal media on shoot multiplication in papaya with TDZ

Basal medium	Co-2		HD		WA	
	% response	No of shoots	% response	No of shoots	% response	No of shoots
MS	76.60±0.58 [d]	11.31±1.95 [b]	73.30±0.58 [d]	11.42±1.98 [b]	73.30±0.58 [d]	11.40±1.72 [c]
B5	90.00±1.00 [b]	11.45±2.40 [b]	93.30±0.58 [b]	11.31±2.56 [b]	90.00±1.00 [b]	11.82±2.02 [b]
Whites	80.00±1.00 [c]	08.59±2.42 [c]	83.30±1.15 [c]	08.92±2.43 [c]	83.30±0.58 [c]	08.96±2.06 [d]
½ MS	66.00±1.15 [e]	07.67±3.19 [d]	63.30±1.53 [e]	08.02±3.87 [d]	66.60±1.15 [e]	07.50±2.92 [e]
MBG	91.00±0.58 [a]	14.51±2.06 [a]	95.53±1.15 [a]	14.62±1.88 [a]	94.43±1.52 [a]	14.57±1.90 [a]

Values with same letter (a-e) in a column are not statistically different at P = 0.05.HD- cv. Honey Dew. Co-2- cv. Co-2 and WA- cv. Washington

When BAP and NAA combinations were tested with all the basal media, almost a similar response was also noted with the explants cultured on MBG basal medium supplemented with BAP and NAA (Table 5.7). In this case, the second best formulation was found to be the MS medium in combination with BAP and NAA. Another important observation was that the buds produced from MBG medium were more dark green in colour as compared to the buds derived from other basal medium in both TDZ and BAP

and NAA supplemented media. This response of papaya explants shows that it needs both high concentration of salts and organic substances along with growth regulators. Therefore, MBG basal medium was used in further carbon experiment.

Table 5.7 Effect of basal media on shoot multiplication in papaya with BAP and NAA

Basal medium	Co-2		HD		WA	
	% response	No of shoots	% response	No of shoots	% response	No of shoots
MS	80.00±1.73 [b]	12.05±1.66 [c]	80.00±1.00 [b]	12.02±2.41 [c]	83.30±0.58 [b]	11.73±1.89 [c]
B5	70.00±0.00 [c]	12.73±1.82 [b]	73.30±1.52 [d]	12.88±1.93 [b]	76.60±1.15 [c]	12.60±1.81 [b]
Whites	70.00±2.00 [c]	08.85±3.12 [d]	76.60±1.52 [c]	08.48±2.71 [d]	73.30±1.52 [d]	08.22±2.26 [d]
½ MS	56.60±2.08 [d]	07.11±2.02 [e]	53.30±2.51 [e]	06.77±2.14 [e]	50.00±2.00 [e]	06.78±1.95 [e]
MBG	95.53±1.52 [a]	14.25±3.90 [a]	93.33±2.64 [a]	14.27±3.93 [a]	94.43±2.08 [a]	14.38±3.81 [a]

Values with same letter (a-e) in a column are not statistically different at P = 0.05.HD- cv. Honey Dew. Co-2- cv. Co-2 and WA- cv. Washington

5.3.1.3 Effect of carbon source on sprouting response of immature embryo axis

The carbon source in the culture medium has been reported to influence proliferation (Enakasha *et al.* 1993) and morphogenesis of cultures (Eapen and George 1993). From our earlier experiments, it can be concluded that MBG was found to be the best basal medium for *in vitro* growth of papaya cultures and TDZ (at 0.5mg/l) and also BAP + NAA (1 and 0.1mg/l) were the optimum hormone concentration for papaya multiple shoot production. Considering these conditions, different carbon sources *viz.* sucrose, glucose, fructose and maltose each at 3% were evaluated for their shoot induction potential and was added to the MBG medium supplemented with TDZ (0.5 mg/l) and BAP and NAA (1 and 0.1mg/l) separately.

Immature embryos when cultured on different carbon sources showed varied response. However, sucrose and maltose were found to be the best carbon source in both the type of hormone supplemented media. Glucose and fructose were found to be not promising carbon source in these experiments. The browning of the embryo was observed within a week of inoculation in both this carbon (Glucose and fructose)

supplemented media. When sucrose is used in the medium as carbon source, it influenced highest number of shoots per explant in all the 3 cultivars tested with both TDZ and BAP and NAA combination. Sucrose has been the most commonly used carbohydrate in papaya tissue culture studies (Rajeevan and Pandey 1986; Mondal *et al.*1990). MBG basal medium with sucrose as carbon source and TDZ (0.5mg/l) as phytohormone resulted in 14.51±2.06, 14.62±1.88 and 14.57±1.90 number of shoots in Co-2, Honey Dew and Washington cultivars respectively (Table 5.8).

Table 5.8 Effect of carbon source on shoot multiplication in papaya with TDZ (0.5 mg/l) in the medium

Carbon	Co-2		HD		WA	
	% response	No of shoots	% response	No of shoots	% response	No of shoots
Sucrose	91.00±0.58[b]	14.51±2.06[a]	95.53±1.15[a]	14.62±1.88[a]	94.43±1.52[a]	14.57±1.90[a]
Fructose	30.00±1.00[d]	04.33±2.10[c]	26.60±1.15[d]	04.33±2.19[c]	33.30±0.58[d]	04.50±2.11[c]
Maltose	93.30±1.15[a]	06.31±1.73[b]	93.30±1.15[b]	06.46±1.79[b]	86.60±0.58[b]	06.28±1.54[b]
Glucose	50.00±0.00[c]	03.3±2.12[d]	43.30±0.58[c]	03.50±2.32[d]	46.60±0.58[c]	03.5±1.93[d]

Values with same letter (a-d) in a column are not statistically different at P = 0.05.HD- cv. Honey Dew. Co-2- cv. Co-2 and WA- cv. Washington

Percent response of the explants was observed to be more with sucrose when used with medium supplemented with BAP and NAA combination. Positive effect of sucrose on shoot regeneration was reported earlier (Sul and Korban, 1998). No significant difference was observed in shoot numbers in all three cultivars when the growth regulator was just replaced with BAP and NAA in the basal medium (Table 5.9).

Table 5.9 Effect of carbon source on shoot multiplication in papaya with BAP and NAA (1.0mg/l and 0.1 mg/l) in the medium

Carbon	Co-2		HD		WA	
	% response	No of shoots	% response	No of shoots	% response	No of shoots
Sucrose	95.53±1.52[a]	14.25±3.90[a]	93.33±2.64[a]	14.27±3.93[a]	94.43±2.08[a]	14.38±3.81[a]
Fructose	36.00±1.15[d]	04.33±2.12[d]	33.30±0.58[c]	04.50±2.25[d]	43.30±1.15[c]	04.50±2.16[d]

Maltose	76.00±0.58 [b]	07.22±1.24 [b]	73.30±0.58 [b]	07.45±1.34 [b]	70.00±0.0 [b]	07.40±1.43 [b]
Glucose	40.00±1.00 [c]	05.33±2.33 [c]	33.30±1.53 [c]	04.83±2.45 [c]	36.60±1.15 [d]	05.00±2.45 [c]

Values with same letter (a-d) in a column are not statistically different at P = 0.05.HD- cv. Honey Dew. Co-2- cv. Co-2 and WA- cv. Washington

Maltose was also observed as equally good in terms of initial cultivar response. Maltose responded best when media is supplemented with TDZ in cv. Co-2 and Honey Dew (Table 5.8). Fructose and glucose at the concentrations tested were found to have an inhibitory role on papaya shoot induction process. However, this result does not tally with the earlier report on effect of fructose on shoot growth (Drew *et al.* 1993). Maltose and glucose did not favour growth of the shoots. With BAP: NAA combination, fructose was the least responsive carbon supplement. On the other hand, glucose responded least when media was supplemented with TDZ. However, root formation was a common observation in the explants kept in fructose and maltose. Shoots growing in glucose were comparatively week and not healthy. Similar view was also put forwarded in papaya tissue cultures by Drew *et al.* 1993.

5.3.2 *In vitro* induction of multiple shoots from shoot tip explant

Although *in vitro* techniques for papaya regeneration through shoot tip culture has been reported earlier (Litz and Conover 1978b; Rajeevan and Pandey 1983), the data on different varieties were found to be inconsistent (Litz 1986). In addition, problems of culture contamination in papaya were also mentioned in earlier reports (Winnaar 1988; Mondal *et al.*1990). In order to overcome these problems, we cultured shoot tips from *in vitro* grown aseptic seedlings and contamination was mostly avoided. This section describes the effect of growth regulators, age of explant, culture vessel and physical state of media on shoot proliferation from shoot tip explant of two very popular papaya cultivars (Honey Dew and Co-2) of India.

5.3.2.1 Effect of growth regulators on multiple shoot formation from shoot tip explant of papaya

In an experiment to asses the effect of various phytohormones, 20 day old shoot tips were cultured on MBG basal media (as MBG medium was found to be the best responsive media in case of both embryo axis and cotyledon explant) supplemented with

wide range of BAP (0.1-2.0mg/l), NAA (0.1,0.5 mg/l), Kin (0.1-2.0mg/l), 2iP (0.1-2.0mg/l) alone or in combination. It was observed that singly none of the hormones were able to induce multiple shoots from the explant (Table 5.10). However, when the cytokinins were combined with auxin NAA, explant responded and produced multiple shoots depending on the concentration of the cytokinin : auxin ratio added to the media.

Table 5.10 Effect of various growth regulators on morphogenetic response of shoot tip explants

Phytohormone	Conc (mg/l)	Response in HD	Response in Co-2
BAP	0.1	NR	NR
	0.5	NR	NR
	1.0	C	C
	2.0	C	C
KIN	0.1	NR	NR
	0.5	NR	NR
	1.0	NR	NR
	2.0	C	C
2ip	0.1	NR	NR
	0.5	NR	NR
	1.0	NR	NR
	2.0	NR	NR
NAA	0.1	C	C
	0.5	C	C

NR- Not responded; C-Callusing at base; HD-Honey Dew; Co-2- Co-2 cv.

Shoot apices were not established in a separate medium but were subcultured at every 2 weeks interval. Proliferation was low in the first transfer but became profuse after the second subculture. The observations recorded here were after 8 weeks of incubation. The induction of multiple shoots from shoot tip explant varied with cytokinin type. Best response was obtained when NAA (0.1 mg/l) was combined with BAP (0.5mg/l), and explants developed 8.64±0.91 and 9.02±0.87 number of shoots in Honey Dew and Co-2 respectively (Fig.5.5 A and B). Next best treatment was found to be the media containing

Kin (0.1mg/l) and NAA (0.1mg/l), with 6.24±1.38 shoots in Honey Dew and 6.64±1.01 shoots in Co-2 (Table 5.11). The explants did not respond in phytohormone free basal medium, which justifies the need of adding growth regulators in papaya tissue culture medium for their proliferation. In our experiment, NAA in low concentration (0.1mg/l) resulted better response with all the cytokinin tested (Fig.5.6A).

Table 5.11 Effect of BAP, Kin and 2ip in combination with NAA on shoot proliferation from shoot tip explants of papaya.

Hormones		HD		Co-2	
BAP	**NAA**	**% response**	**Shoots/explant**	**% response**	**Shoots/explant**
0.1	0.1	36.33±3.52 [e]	03.94±1.88 [d]	36.55±3.23 [e]	04.43±1.55 [d]
0.5	0.1	91.77±2.98 [a]	08.64±0.91 [a]	91.55±2.99 [a]	09.02±0.87 [a]
1.0	0.1	46.33±4.35 [c]	05.27±0.63 [c]	46.88±4.48 [c]	05.61±0.97 [c]
2.0	0.1	23.11±2.45 [g]	03.12±1.03 [e]	23.22±2.27 [g]	03.72±1.34 [e]
0.1	0.5	23.55±2.18 [g]	01.44±0.58 [h]	23.33±0.76 [g]	01.65±0.71 [g]
0.5	0.5	30.77±2.37 [f]	05.55±0.96 [b]	31.33±2.06 [f]	05.65±0.95 [b]
1.0	0.5	65.66±4.00 [b]	02.87±0.97 [f]	66.44±2.92 [b]	02.57±0.75 [f]
2.0	0.5	43.11±2.78 [d]	01.56±0.76 [g]	46.22±2.79 [d]	01.35±0.56 [h]
Kin	NAA				
0.1	0.1	85.88±3.75 [a]	06.24±1.38 [a]	86.11±3.44 [a]	06.64±1.01 [a]
0.5	0.1	51.33±3.21 [b]	04.13±1.14 [b]	52.44±3.28 [b]	3.91±1.01 [b]
1.0	0.1	16.44±4.40 [d]	03.46±0.56 [d]	15.99±2.93 [e]	3.27±0.48 [d]
2.0	0.1	09.00±0.70 [a]	02.90±0.59 [e]	09.66±1.03 [g]	3.02±0.72 [e]
0.1	0.5	16.66±2.08 [d]	03.99±1.22 [c]	18.77±3.34 [d]	3.55±1.03 [c]
0.5	0.5	35.66±2.11 [c]	01.67±0.58 [h]	36.22±1.89 [c]	1.84±0.50 [g]
1.0	0.5	13.88±2.61 [e]	02.02±0.89 [f]	14.11±2.45 [f]	1.85±0.69 [f]
2.0	0.5	09.88±2.19 [f]	01.90±0.86 [g]	8.44±2.55 [h]	1.72±0.87 [h]
2ip	NAA				
0.1	0.1	09.55±1.71 [c]	03.25±1.12 [b]	09.88±1.21 [b]	03.35±1.01 [b]
0.5	0.1	05.77±2.10 [d]	02.78±0.72 [c]	06.0±2.00 [e]	02.97±0.74 [c]
1.0	0.1	05.11±2.38 [f]	03.45±0.97 [a]	05.66±2.18 [f]	03.57±0.73 [a]

2.0	0.1	02.11±0.85 [h]	01.91±0.53 [f]	02.22±0.85 [h]	02.25±0.74 [d]
0.1	0.5	19.11±1.78 [a]	02.43±0.55 [d]	18.22±2.35 [a]	02.12±0.58 [f]
0.5	0.5	10.22±1.53 [b]	01.46±0.56 [g]	09.22±1.71 [c]	01.56±0.40 [g]
1.0	0.5	05.55±1.38 [e]	02.23±1.58 [e]	06.11±1.74 [d]	02.34±1.51 [e]
2.0	0.5	03.11±1.68 [g]	01.46±0.56 [g]	02.55±1.40 [g]	01.22±0.54 [h]

Values with same letter (a-h) in a column are not statistically different at P = 0.05.

HD- cv. Honey Dew. Co-2- cv. Co-2

The differential effect of various phyto hormones have been reported in papaya by many authors (Drew and Smith 1986; Rajeevan and Pandey 1983; Islam *et al.* 1993).

BAP was the most effective phytohormone in all the cases along with NAA

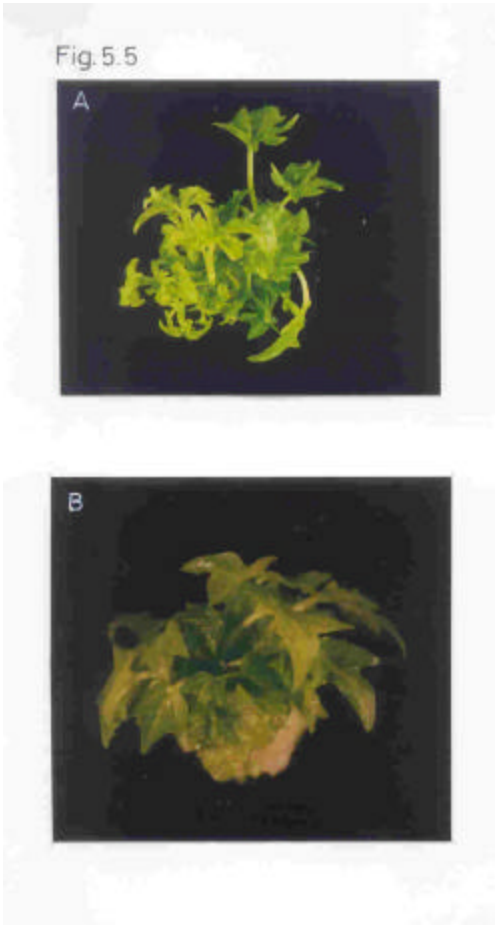


Fig. 5.5

A. Proliferation of shoot tip explant of cv. Honey Dew in presence of BAP (0.5mg/l) and NAA (0.1 mg/l) in MBG basal medium

B. Proliferation of shoot tip explant of cv. Co-2 in presence of BAP (0.5mg/l) and NAA (0.1 mg/l) supplemented MBG *basal medium*

indicating a cytokinin specificity for multiple shoot induction in explants of papaya.

5.3.2.2 Effect of seedling age on shoot tip proliferation

The age of the explant was also found to influence the morphogenetic response (Kameya and Wudholm 1981; Schroeden and Stimart 1999). In our study, the induction of multiple shoots from explants varied with seedling age. Three types of explants (7 day old, 20 day old and 30 day old explant) were used in the study (Fig.5.6B). All these shoot tips were inoculated in MBG medium supplemented with BAP +NAA concentration at 0.5 and 0.1 mg/l. Thirty day old seedling tips evoked maximum shoots per explant (14.53±0.74 and 15.19±0.65) (Table 5.12) in Honey Dew and Co-2 respectively. Least response was observed in explant derived from 7 day old seedlings. This shows that 30 day old papaya shoot tips were the most responsive explant when cultured on MBG medium supplemented with hormonal combination of BAP (0.5mg.l) and NAA (0.1mg/l).

Table 5.12 Effect of seedling age on shoot multiplication from shoot tip explants of papaya.

Seedling age (days old)	HD		Co-2	
	% response	Shoots/explant	% response	Shoots/explant
7	100	03.11±1.10 [a]	100	03.22±0.91 [a]
20	100	08.76±1.86 [b]	100	08.98±1.53 [b]
30	100	14.53±0.74 [c]	100	15.19±0.65 [c]

Values with same letter (a-c) in a column are not statistically different at P = 0.05.

HD- cv. Honey Dew. Co-2- cv. Co-2

5.3.2.3 Effect of culture vessel on shoot tip proliferation

Effect of two types of culture vessel, test tube and glass bottles were tested to see their effect on shoot tip proliferation of papaya explant. In our experiment, a marked difference was observed in the number of multiple shoots obtained from 30 day old seedling explants, cultured in test tube and in glass bottles. Shoot tip explants excised from 30 day old seedlings and cultured on glass bottle in medium supplemented with BAP and NAA (0.5mg/l and 0.1 mg/l) showed an increased induction (14.53±0.74 and

15.19±0.65 in Honey Dew and Co-2 respectively) of multiple shoots (Fig.5.7A). In contrast, explants cultured in test tubes and cultured on same medium resulted 4.94±1.06, 4.38 ±0.70 shoots/explant in Honey Dew and Co-2 respectively (Fig.5.7B) (Table 5.13). This effect is probably due to the large amount of medium (50 ml compared to 20ml in test tube) and larger airspace (350 cm^3 compared to 30 cm^3 in test tubes) available to the seedlings. The positive influence of larger culture vessel was reported earlier (McClelland and Smith 1990). Although our study did not measure the gaseous exchange in test tubes or bottles, the influence on shoot growth rate of the type of culture vessel and enclosures due to varying amount of ethylene / carbon dioxide release has been well documented (De proft *et al.* 1985).

Table 5.13 Effect of culture vessel on shoot proliferation from shoot tip explant of papaya

Culture vessel	HD		Co-2	
	% response	Shoots/explant	% response	Shoots/explant
Test tube	100	04.94±1.06 [a]	100	04.38±0.70 [a]
Glass bottle	100	14.53±0.74 [b]	100	15.19±0.65 [b]

Values with same letter (a-b) in a column are not statistically different at P = 0.05. HD- cv. Honey Dew. Co-2- cv. Co-2

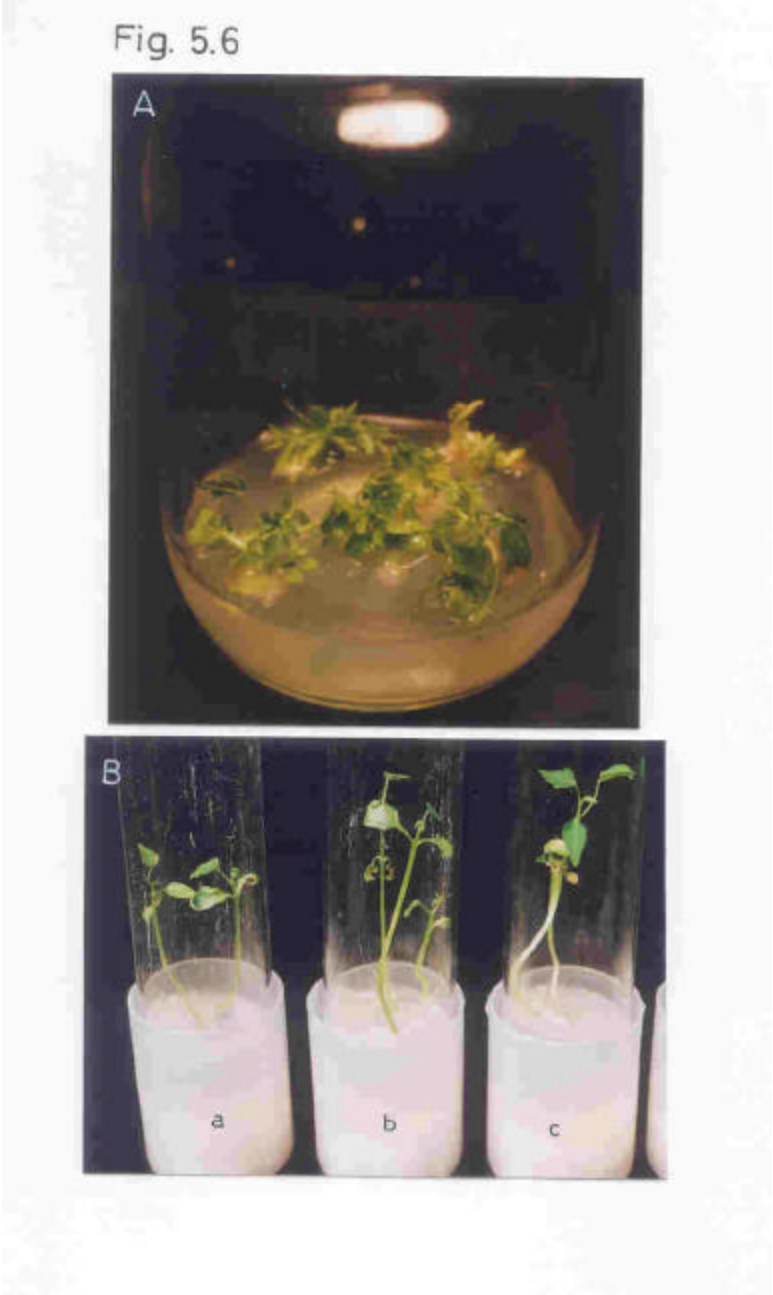


Fig. 5.6

A. Proliferation of shoot tip explant after 10 days of incubation in media containing NAA (0.1mg/l)

B. Ten day, twenty days and thirty days old seedling used for shoot tip proliferation experiment, a-10days b-20 days c-30 days old seedling

5.3.2.4 Effect of physical state of media on shoot proliferation from shoot tip explant of papaya

In this experiment, effect of solid (media with agar) and liquid media (media without agar) were tested in induction of multiple shoots from shoot tip explant. It is seen that physical state of the medium affects positively the number of shoots produced per explant in papaya. The state of media also affects the general appearance of the shoots produced.

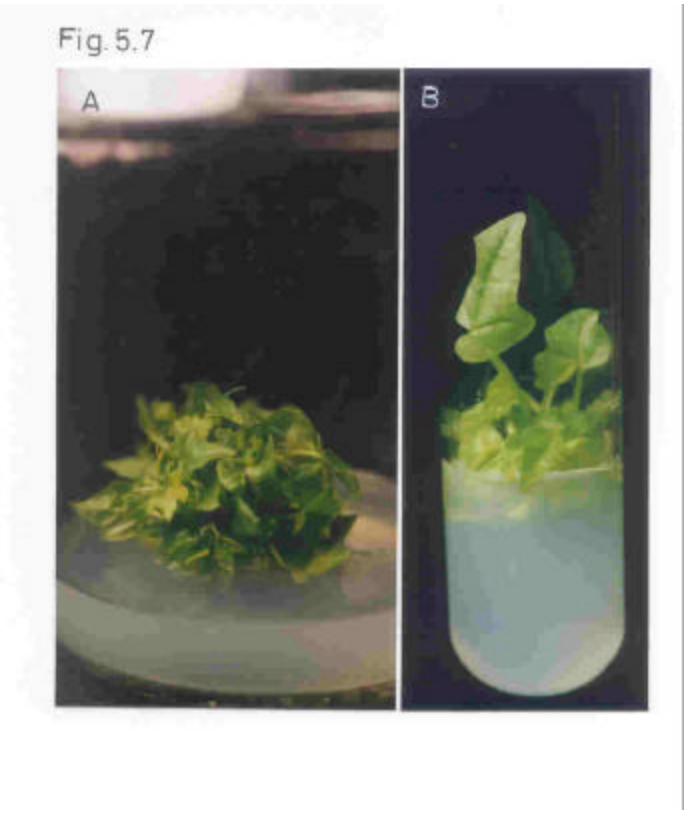


Fig. 5.7

A. Proliferation of a single shoot tip in glass bottle, after 45 days of incubation in MBG media containing BAP and NAA (0.5mg/l and 0.1 mg/l) (cv. Honey Dew)

B. Proliferation of a single shoot tip in test tube, after 45 days of incubation in MBG media containing BAP and NAA (0.5mg/l and 0.1 mg/l) (cv. Honey Dew)

Semisolid media could favour on an average 14.53±0.92 and 15.19±0.65 number of shoots in cultivars Honey Dew and Co-2 (Fig.5.8A), whereas explants when cultured on liquid media developed 19.06±1.24 and 20.00±1.27 number of shoots (Fig.5.8B) in Honey Dew and Co-2 respectively (Table 5.14).

Table 5.14 Effect of physical state of the medium on shoot tip proliferation in papaya

Media state	HD		Co-2	
	% response	Shoots/explant	% response	Shoots/explant
Solid	100	14.53±0.74 [a]	100	15.19±0.65 [a]
Liquid	100	19.06±1.24 [b]	100	20.00±1.27 [b]

Values with same letter (a-b) in a column are not statistically different at P = 0.05. HD- cv. Honey Dew. Co-2- cv. Co-2

Induction of large number of shoots in liquid medium as compared to semisolid medium has been reported earlier (Kim *et al.*1997). Compared to the shoots produced in the solid media, the shoots in liquid media were healthy, deep green in colour and much more elongated. In our experiment, liquid media showed an improvement over semi solid medium not only in number of shoot produced but also on the number of days required to produce the shoots. In liquid media, shoot initials could be observed after 15-20days whereas shoot initials were noticed after 4 weeks of culture in case of semi solid media. In earlier studies (Shlesinger *et al*. 1987;Castillo *et al*. 1998b), it has been observed that liquid media influence the growth of papaya cultures. The probable reason of this may be that the liquid medium facilitates better aeration, easy diffusion and flow of nutrients and growth regulators by capillary forces to the explants as also pointed out previously (Constabel and Shyluk 1994).

5.3.4 Elongation of shoots

After 4 to 5 weeks of culture, the regenerated shoots obtained from the immature embryo axis were transferred to MBG medium free of phytohormone. Elongation of shoots was observed after another 3-4 weeks of incubation. However, media supplemented with higher TDZ concentrations were found to be inhibitory for elongation of shoots and all the shoots formed were stunted. The problem of shoot elongation was overcome by transferring the shoot cultures (of 5-6 shoot buds) to MBG medium

supplemented with GA_3 (1-5.0 mg/l) for 15 days. Elongation of shoots with an average length 3.51±0.29 cm was observed on MBG medium supplemented with 2 mg/l GA_3 (Table 5.15) (Fig. 5.9A). Regenerated shoots from BAP and NAA supplemented medium can be elongated in phytohormone free basal medium (Fig. 5.9B). Well developed elongated shoots with 1-4 cm length were separated and kept for rooting (Fig. 5.10A). Morphologically, the regenerated shoots did not show any visual abnormalities. Shoot elongation was also evaluated in liquid MBG medium supplemented with GA_3 (2mg/l) (Fig.5.10B).

Fig. 5.8

A. *Proliferation of shoot tip in MBG semisolid media containing BAP and NAA (0.5mg/l and 0.1 mg/l) after 45 days of incubation (cv. Co-2)*
B. *Proliferation of shoot tip in liquid MBG semisolid media containing BAP and NAA (0.5mg/l and 0.1 mg/l) after 45 days of incubation (cv. Co-2)*

Fig. 5.9

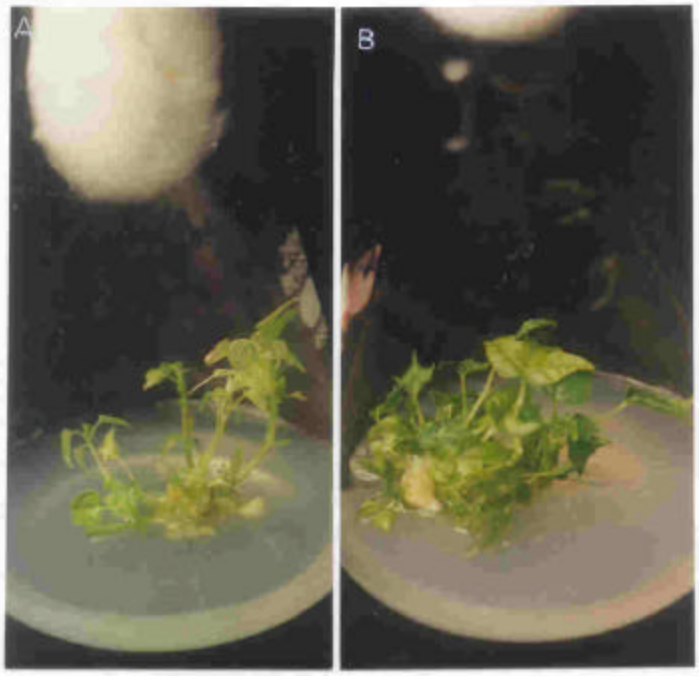


Fig. 5.9

A. Elongation of shoots (obtained from 1.5mg/l TDZ) in media supplemented with GA_3 (2.0 mg/l)

B. Elongation of shoots (obtained from 1.0mg/l BAP and NAA 0.5 mg/l) in phytohormone free basal medium

Table 5.15 Effect of GA_3 on shoot bud elongation in papaya

GA_3 conc.(mg/l)	**% shoot masses showing elongation**	**Shoots elongated per shoot mass**	**Length of elongated shoots**
0	00.00±0.00 [a]	00.00±0.00 [e]	00.00±0.00 [e]
1	25.33±4.50 [c]	02.00±1.00 [c]	02.65±0.21 [d]
2	91.33±1.15 [a]	06.66±1.15 [a]	03.51±0.29 [a]
3	48.33±2.88 [b]	03.33±0.58 [b]	02.70±0.27 [c]
4	18.66±3.21 [d]	01.66±0.58 [d]	02.75±0.07 [b]
5	04.33±2.08 [e]	01.66±1.15 [d]	02.70±0.14 [c]

Values with same letter (a-e) in a column are not statistically different at P = 0.05.

Shoots cultured in the liquid medium exhibited a higher increase in length. For elongation of the *in vitro* shoots, use of GA_3 is well depicted (Schnabelrauch and Sink

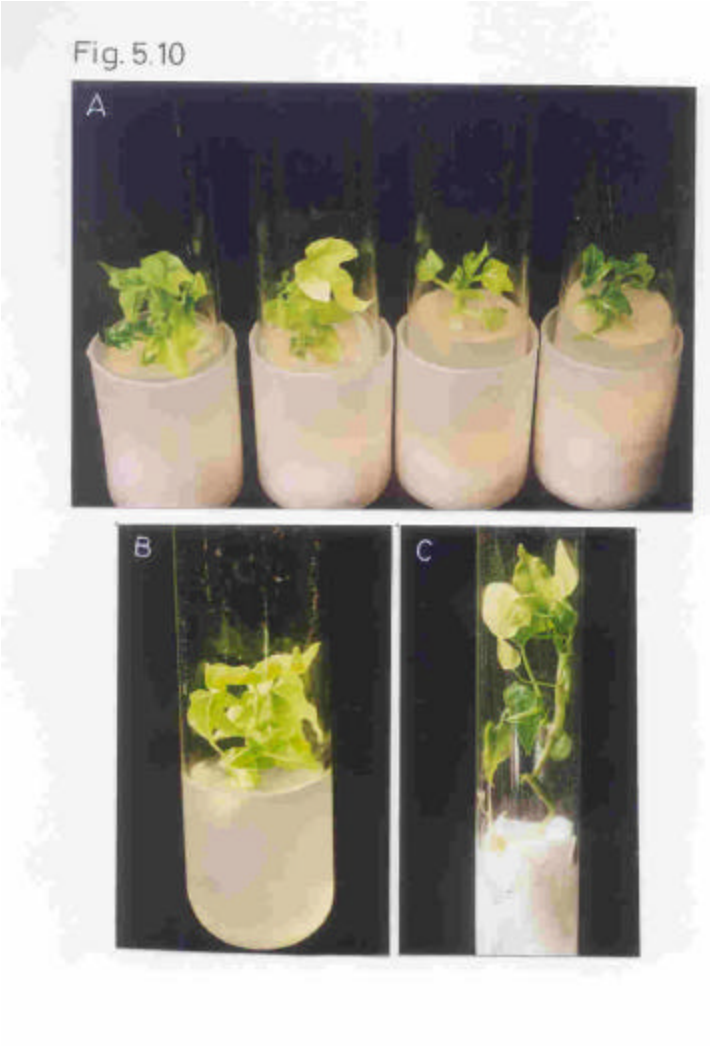


Fig. 5.10

A. Elongated shoots (obtained from shoot tip proliferation) kept for rooting

B. Elongation of shoots (obtained from shoot tip proliferation) in phytohormone free MBG basal medium, cv. Honey dew

C. Shoot elongation (obtained from TDZ supplemented media) in presence of GA_3 (2.0 mg/l) in liquid MBG basal medium, cv. Co-2.

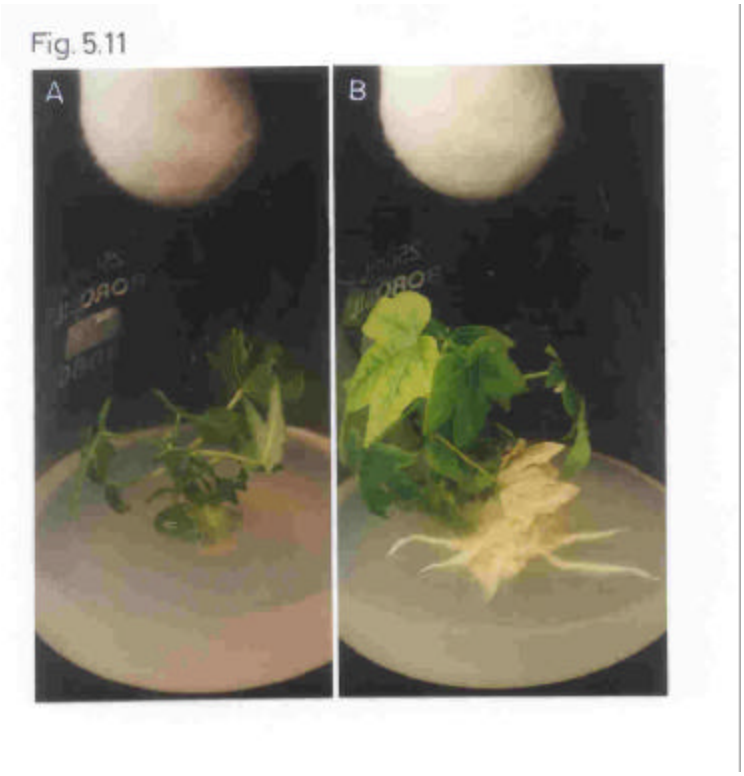


Fig. 5.11

A. Elongated individual shoots kept for rooting in MBG basal media supplemented with IBA (1-4 mg/l) cv.Co-2

B. Rooted shoot in presence of IBA (3.0 mg/l) in the rooting medium cv. Honey Dew

Fig. 5. 2

Fig. 5.12

A. Rooted plantlets obtained from MBG medium supplemented with TDZ, a-Honey Dew; b- Co-2

B. Rooted plantlet of cv. Washington obtained from BAP and NAA supplemented MBG medium

C. Rooted plantlet of cv. Co-2 obtained from shoot tip proliferation

1979; Baburaj *et al*. 1987; Sujatha and Reddy 1998). It is also reported that GA_3 facilitate elongation of buds initiated by TDZ treatment (Bhagwat *et al*. 1996).

Similarly the shoots obtained from shoot tip culture can be elongated on MBG basal medium only without addition of any phytohormone (Fig.10c). Elongation of shoots on hormone free medium has been obtained in papaya (Litz and Conover 1977; Hossain *et al*. 1993) and also in *Clianthus formosus* (Taji and Williams 1989), *Leucopogon obtectus* (Bunn *et al*. 1989) and a hybrid larch (Brassard *et al*. 1996).

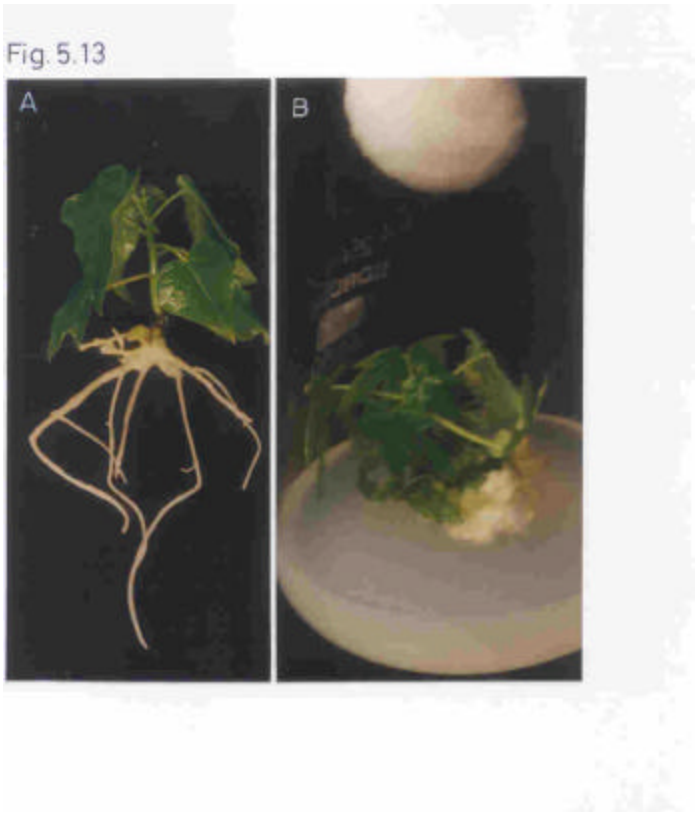


Fig. 5.13

A. Normal rooting of plantlet in presence of 3 mg/l IBA, cv. Washington

B. Callusing of the shoots in presence of high concentration of IBA (4 mg/l)

5.3.5 Rooting

Well developed and elongated shoots of about 2 -5 cm in height were excised after 15-20 days and transferred to half concentration of MBG medium supplemented with 1-4 mg/l IBA and 3% sucrose to achieve rooting of the shoots (Fig.5.11A). Ten shoots in each treatment were used. These shoots developed roots after 2 weeks of incubation in all the media tested (Fig. 5.11B). MBG medium with 3 mg/l IBA induced maximum percent rooting (93.33%) (Fig. 5.12 A and B). Similar to our study, the effect of IBA on papaya root formation was achieved earlier (Mondal *et al.*1990). Phytohormone free basal medium served as control. Lowest rooting response was observed on control media followed by 1 mg/l IBA (Table 5.16).

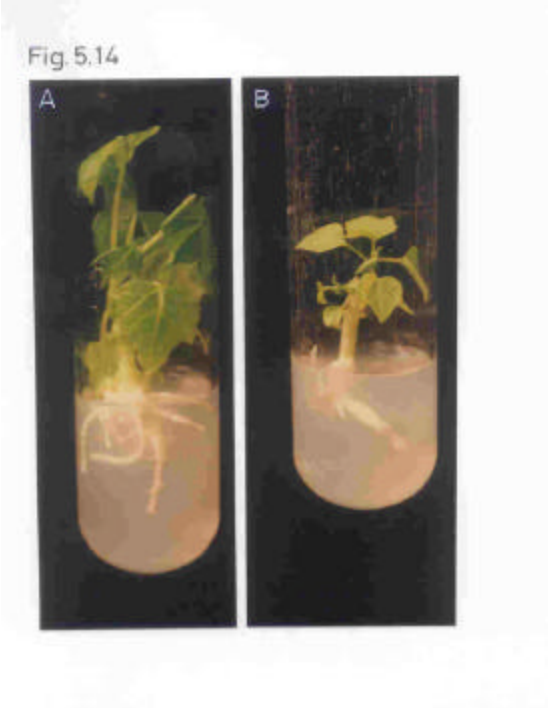


Fig. 5.14

A. Rooting of shoot in presence of IBA (3.0 mg/l), cv. Honey Dew

B. Rooting of shoot in IBA free MBG basal medium cv. Honey Dew

Table 5.16 Effect of IBA on rooting of shoots in papaya

IBA conc. (mg/l)	% shoot forming root	Days for rooting
0	23.33±0.58 [e]	14.66±1.17 [a]
1	33.33±0.58 [d]	14.33±0.23 [a]
2	73.33±0.57 [c]	14.00±0.70 [a]
3	93.33±0.58 [a]	14.33±0.47 [a]
4	80.00±1.00 [b]	15.00±0.00 [a]

Values with same letter (a-f) in a column are not statistically different at P = 0.05.

Sporadic callusing was also observed in shoots cultured in media containing IBA (4 mg/l) (Fig. 5.13B). In general, shoots produced less number of roots in media containing IBA 1, 2 and 4 mg/l. It was observed that shoots rooted with IBA were more vigorous as compared to the shoots rooted in phytohormone free MBG medium. (Fig.5.14 A,B). Presence of IBA in the rooting medium of papaya was found to be beneficial as reported earlier, however, high concentartion of the auxin can also inhibit further growth and development of root as reported (Drew 1987).

The elongated shoots obtained from shoot tip cultures were also rooted on a medium containing half strength MBG medium supplemented with 3 mg/l IBA (medium already standardized for rooting of shoots from immature embryo) (Fig. 5.12C). This showed that IBA is beneficial for optimum root induction of papaya plantlet.

5.3.6 Hardening and transfer to pots

For acclimatization, the rooted shoots (3-5 cm length) were washed with sterile water to remove the agar sticking to them and then transferred to 8 cm pots, covered with plastic bag for a week to prevent desiccation (Fig.5.15 A). Hardened plantlets were then transferred to green house. The rooted shoots obtained from embryo axis explant after transfer to pots survived and hardened (76%) under greenhouse condition and thereafter planted on pots before transferring to field (Fg. 5.15 B). The survival rate of the shoot tip derived plantlets was found to be 85% after hardening. Normal growth was observed in all hardened plants.

Fig. 5.15

A. Hardening of tissue cultured plants in 8 cm pots

B. Papaya plants after hardening ready to transfer in field

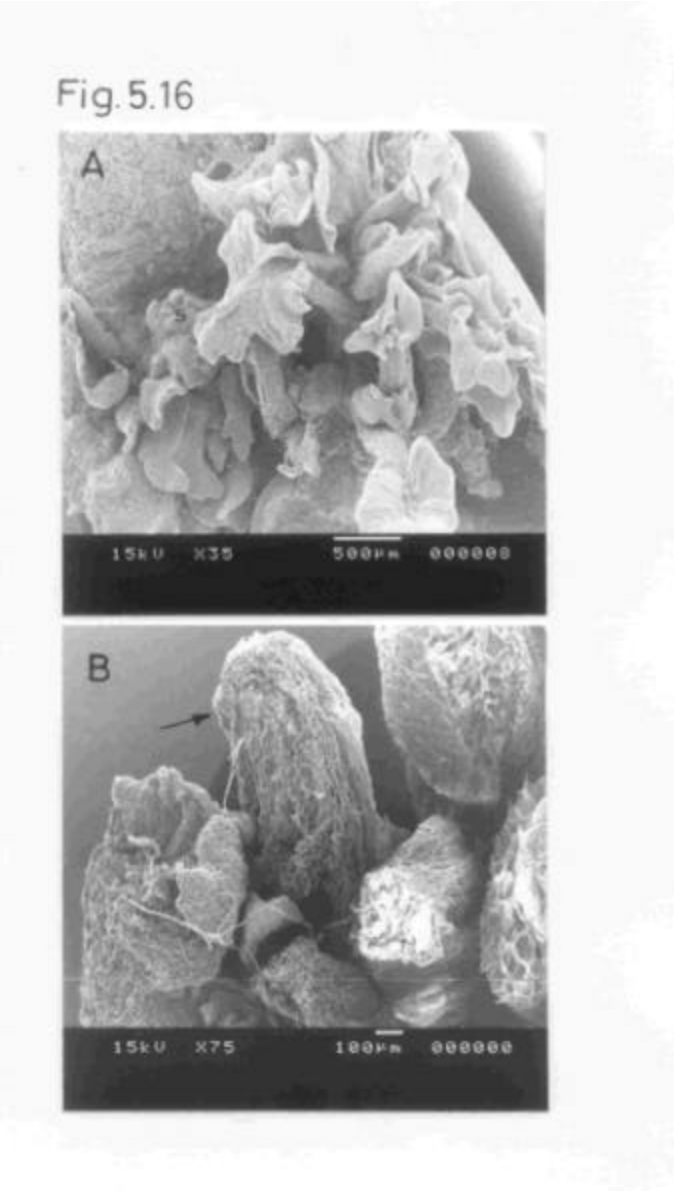


Fig. 5.16

A. Scanning electron micrograph (SEM) study showing direct formation of shoot buds and shoots form immature embryo axis of papaya, s-shoots, b-buds

B. Arrow indicates an enlarged view of single shoot bud (through SEM study) originating from immature cotyledon explant

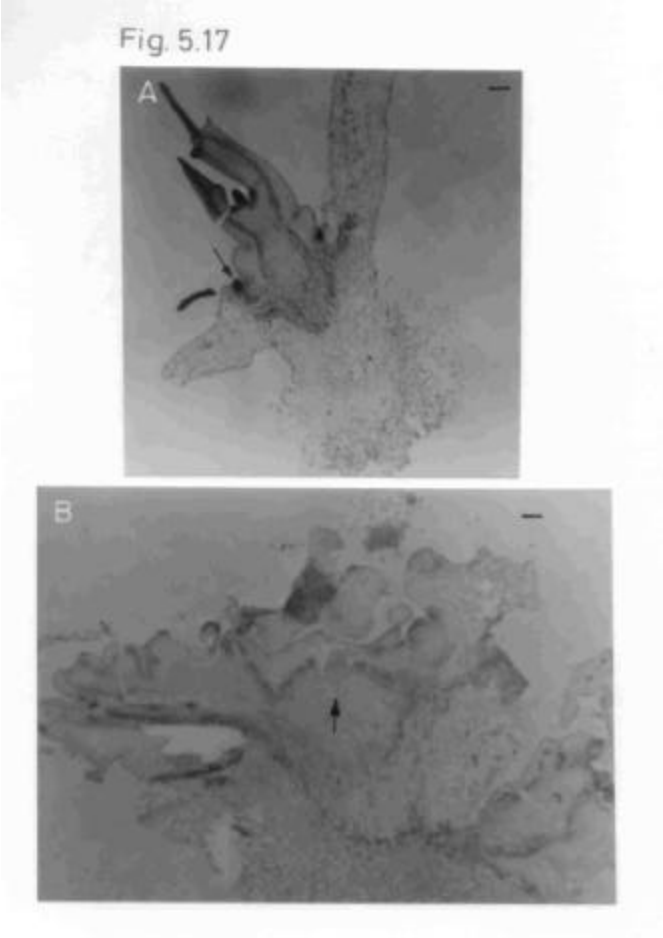


Fig. 5.17

A. Longitudinal section of an embryo axis explant showing direct origin of shoot buds from shoot pole of embryo axis after 15 days of incubation. Arrow indicates the emerging shoot bud. Bar=350 µm

B. Longitudinal section of an immature embryo showing the meristematic activity observed in the embryo explant after three weeks of incubation. Arrow indicates meristematic pockets. Bar=37 µm

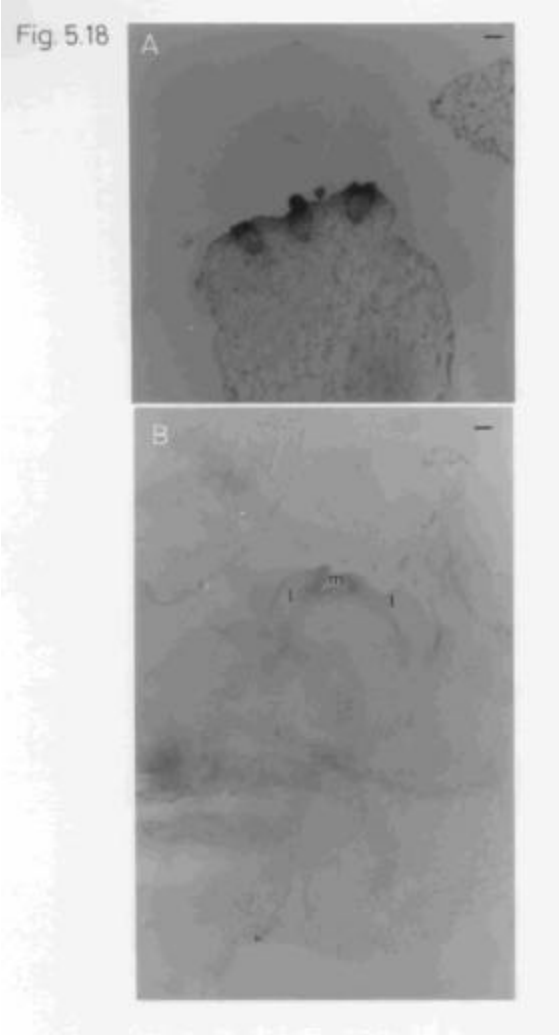


Fig. 5.18

A. An enlarged view of meristematic pockets in the embryo explant after 3 weeks of culture. Bar=7.16 µm

B. A longitudinal section of a developing shoot bud showing the shoot meristem with leaf primordia. m-meristem l-leaf primordia. Bar=100 µm

5.4 Conclusion

Plant regeneration via multiple shoot development from embryo axis of papaya has not been reported so far. Though there are few reports on plant regeneration from shoot tip explants of papaya, the effects of various culture conditions like seedling age

influence of culture vessels, type of phytohormones etc on shoot tip explants for high efficiency shoot development were also not reported earlier. In the present study, efforts were made to develop plant regeneration protocol from immature embryo and shoot tip explant of some commonly cultivated papaya cultivars.

Maximum induction of multiple shoots (14 shoots per explant) could be achieved from immature embryos (both from axis and cotyledons) when cultured in MBG (MS salts + B5 vitamins + 2 mg/l glycine) medium supplemented with TDZ (0.5mg/l) and also in media containing BAP +NAA (1.0+0.1mg/l). Among the basal media tested, MBG (MS salts + B5 vitamins + 2 mg/l glycine) medium was found to be the most suitable basal medium for multiple shoot induction from papaya explants. Also, sucrose was found to be the most effective carbohydrate source for *in vitro* papaya shoot formation compared to other carbohydrates evaluated. Elongation of the shoots were obtained either in phytohormone free medium or MBG medium supplemented with GA_3 (2.0 mg/l). The best rooting response (93.33%) of the *in vitro* shoots was achieved on MBG basal medium supplemented with IBA (3mg/l). Survival of tissue cultured plants was 76% from embryo axis explants, after hardening under greenhouse conditions. The present methods described here could be of use in *in vitro* culture of papaya as the process is rapid and very efficient. Also, use of embryo axis explant for multiple shoot development has several additional advantages. Due to its smaller size, it is easy to use in both *Agrobacterium* and particle bombardment mediated transformation technique. It takes less time to develop shoots. Because of these benefits, embryo axis is now being preferred over other explant for transformation experiments.

In another approach, shoot proliferation from shoot tip cultures of cultivars Honey Dew and Co-2 were undertaken. Maximum number of shoots were obtained in the explants cultured on MBG media containing BAP (0.5mg/l) and NAA (0.1mg/l) in both the cultivars. Explants from 30 day old seedling produced maximum number of shoots (19 shoots in Honey Dew and 20 shoots in Co-2) in the MBG liquid medium supplemented with BAP (0.5mg/l) and NAA (0.1mg/l). In our study, a marked difference was observed when the influence of culture vessels was evaluated for induction of multiple shoots Explants cultured on glass bottle produced higher number of shoots (Honey Dew-14.53 and Co-2-15.19) as compared to those cultured in test tubes (Honey

Dew-4.94 and Co-2-4.38). Also liquid medium has favored the maximum induction of shoots (Honey Dew-19.06 and Co-2-20.00) from explants compared to solid medium (Honey Dew-14.43 and Co-2-15.19). Elongation of the shoots was achieved on phytohormone free medium. Rooted shoots survived (85%) after hardening in green house.

Part of this chapter has been communicated as paper to Biologia Plantarum

CHAPTER 6

TRANSFORMATION STUDIES IN PAPAYA

Section A

6.1 Introduction

Genetic manipulation of plants has been an ongoing science since prehistoric times, when early farmers along the Euphrates began carefully selecting and maintaining seed from their best crops to plant for the next season. Early people also bred plants, and modern crops are a result of thousands of years of genetic manipulation (O'Neal 2001).

Conventional plant breeding, however, has its own limitations. It depends on sexual compatibility and often takes 10-15 years to release a new variety due to extensive backcrossing (Pauls 1995). Due to unsuccessful crosses and narrow gene pool available within a species, genetic engineering is now a days, used as an additional tool to crop improvement programs being studied for increasing the qualitative and quantitative food production.

Genetic transformation is a process through which genetic materials isolated from one organism can be introduced into and expressed in another organism with different genetic background. This process involves several distinct stages, namely insertion, integration, expression and inheritance of the newly introduced gene in the host genome. In plant, this technology not only has potential to achieve crop improvement with a more rapid and precise manner than the conventional breeding programs, but also has became an indispensable enabling tool for further dissection and understanding of the plant species.

Genetic engineering has allowed explosive expansion of our understanding in the field of plant biology and provides us with the technology to modify and improve crop plants. A remarkable progress has been made in the development of gene transfer technologies (Gasser and Fraley 1989) which ultimately have resulted in production of a large number of transgenic plants both in dicots and monocots. Potential benefits from these transgenic plants include higher yield, enhanced nutritional values, reduction in pesticides and fertilizer use and improved control of soil and water pollutants. Some of the important characters like resistance to herbicide (Smith 1994), disease (Smith 1994), insect (Perlak *et al*. 1990), high protein content (Habben and Larkins 1995), cold tolerance (Georges *et al*. 1990), fruit quality (Fray and Grierson 1993), biodegradable

plastics (Poirier *et al*. 1995), antibodies and vaccines (Mason *et al*. 1992) etc. have been incorporated in the genetically engineered plants.

6.1.1 History of genetic transformation

It took more than 2000 years to detect the causal principle of the crown gall disease after it was first described by Aristotle's and Theophrastus (Siemens and Schieder 1996). Smith and Townsend (1907) were the first to report that Agrobacterium tumefaciens is the causative agent of the widespread neoplastic plant disease crown gall. Since then a large number of scientists throughout the world have focused their research to understand the molecular mechanism of crown gall induction. The soil bacterium A. tumefaciens and A. rhizogenes are considered as natural genetic engineers due to their ability to transfer and integrate DNA into plant genomes through a unique integrative gene transfer mechanism (Jouanin et al. 1993).

It was only in 1983 that scientist inserted the first foreign genes into Petunia and tobacco (Kung 1984). Agrobacterium-mediated gene transfer became the method of choice due to convenience and high probability of single copy integration. Independently, several transgenic tobacco plants were produced to express foreign genes engineered by the Agrobacterium tumefaciens vectors (Murai et al.1983; Horsch et al. 1984; De Block et al. 1984). Initially successes in genetic transformation were limited to the species of Solanaceae especially tobacco (N. tavacum L.). However, this changed the situation dramatically in late 80's and early 90's and resulted in transformation of a wide range of plants for agronomically important traits (Songstad et al.1995) using genetically engineered avirulent strains of Agrobacterium as vectors (Herrera-Estrella et al. 1983).

Since the initial successes in the Agrobacterium mediated transformation were mostly confined to dicotyledonous plants, concerted efforts were made to look for alternative methods of gene transfer. Method of direct gene delivery into protoplasts was the next development in genetic transformation (Draper 1982), Further many more techniques such as macroinjection (Zhou et al. 1983, 1988), soaking pollen in DNA solution (Ohta1986), pollen transformation via pollentube pathway (Luo and Wu 1988), microinjection (Neuhaus and Spangenberg 1990**),** silicone carbide fibres (Kaeppler et al. 1990), electroporation (DeKeyser et al.1990), sonication (Joersbo and Brunstedt 1990), electrophoresis (Griesbach and Hammond 1993), laser mediated gene transfer (Guo et al.

1995) have been developed. However, none of these approaches has, so far, been developed into a reproducible universal gene transfer technique (Potrykus 1995).

The next breakthrough in genetic transformation was the development of biolistic (Particle bombardment) transformation approach (Klein et al. 1987; Sanford 1988). The process involves coating DNA on tiny metal particles and shooting these into plant tissues, which not only led to the efficient production of transgenic model plants but also opened a route for genetic engineering of major crop plants (Potrykus 1995).

6.1.2 *Agrobacterium* mediated gene transfer technique:

Agrobacterium is a Gram-negative, soil-dwelling bacterium, which infects plant cells near wounds, usually at the junction between the root and stem (crown) in a wide range of plant species. *Agrobacterium*–mediated gene transfer involves incubation of cells or tissues with the bacterium (cocultivation), followed by regeneration of plants from the transformed cells. For plant species that are readily amenable to tissue culture, *Agrobacterium* mediated gene transfer the first widely adopted methods of developing transgenic plants, remains the most popular technique. Probably the greatest advantage of the system is that it offers the potential to generate transgenic cells at relatively high frequency, without a significant reduction in plant regeneration rates. The system is simple, inexpensive and in many cases efficient. Moreover the DNA transferred to the plant genome is defined, it does not normally undergo any major rearrangements and it integrates into the genome as a single copy (Walden and Wingender 1995).

Agrobacterium tumefaciens possess a tumor inducing (Ti) plasmid responsible for the tumor formation (Zupan and Zambryski 1995) whereas *A. rhizogenes* possess a root inducing (Ri) plasmid which is responsible for DNA transfer and the resulting hairy root formation (Tepfer 1984). During infection, the bacterium transfers a small section of its own genetic material (T-DNA) into the genome of the host plant's cell (Zambryski 1992). Once inserted, the bacterial genes are expressed by infected cells of that plant. During the infection process, first the plant cell begins to proliferate and form tumors and then synthesize an arginine derivative called opine. The opine synthesized is usually nopaline or octopine depending on the strain involved. These opines are catabolized and used as energy sources by the infecting bacteria. By understanding and manipulating this process

of infection or transformation, scientists have been able to harness these powerful and sophisticated vectors to transfer specific cloned genes of major importance.

Initially, monocotyledons were considered outside the host range of *Agrobacterium*. However, advances in understanding of the biology of the infection process, availability of gene promoters suitable to monocotyledons (Wilmink *et al*. 1995) as well as selectable markers have improved transformation of monocotyledons (Smith and Hood 1995). Transgenic plants of rice (Hiei *et al*. 1994) and maize (Ritchie *et al*. 1993) have been produced via *Agrobacterium*-mediated transformation.

So far, large number of plant species have been transformed by *Agrobacterium*-mediated transformation technique (Siemens and Schieder 1996). However, success of *Agrobacterium*–mediated transformation depends on the cultivar (Robinson and Firoozabady 1993), the choice of explants (Robinson and Firoozabady 1993; Jenes *et al*. 1993) the delivery system, the *Agrobacterium* strain (Gelvin and Liu 1994), the conditions of cocultivation, the selection method and the mode of plant regeneration. *Agrobacterium* cocultivation has been successfully used for the transformation of leaves, roots, hypocotyls, petioles, cotyledons (Zambryski 1992; Hooykaas and Beijersbergen 1994), pollen-derived embryos (Sangwan *et al*. 1993), seeds (Feldmann and Marks1987) and even plants (Chang *et al*. 1994).

T-DNA of *Agrobacterium* is a small section of the plasmid DNA, about 23 kb in size, which makes up about 10% of the Ti or Ri plasmids. This stretch of DNA is flanked by 25bp repeated sequences, which are recognized by the endonucleases encoded by the vir genes. Within the T-DNA, two distinct regions TL and TR have been identified. The T-DNA of nopaline strains can integrate as a single segment, whereas octopine strains frequently integrate as two segments, TL and TR. TL carries the genes controlling auxin and cytokinin biosynthesis and is always present when tumors are formed. Failure of TR to integrate, results in the loss of opine biosynthesis (Webb and Morris 1992). The *vir* (virulence) region of Ti plasmid contains the genes, which mediate the process of T-DNA transfer. Vir gene action generates and processes a T-DNA copy and facilitates T-DNA movement out of the bacterium and into the plant cell. Helper plasmids for non-oncogenic plant transformation have been developed to utilize the vir gene functions with T-DNAs containing genes of choice (Hood *et al*. 1993).

The removal of the oncogenes from the Ti plasmid results in disarmed strains of *A. tumefaciens* (Klee *et al*. 1987). The oncogenes of *Agrobacterium* are replaced by reporter genes / screenable marker genes (e.g. β-glucuronidase gene (gus), luciferase (luc) gene for analyzing gene expression. Genes conferring resistance to antibiotics (e.g. neomycin phosphotransferase II (nptII), hygromycin phosphotransferase (hpt), phosphinothricin acetyl transferase (bar) are used to allow selection between transgenic and non transgenic cells. Also oncogenes have been replaced by genes of economic importance (McElroy and Brettel 1994).

Plants are usually transformed with relatively simple constructs, in which the gene of interest is coupled to a promoter of plant, viral or bacterial origin. Some promoters confer constitutive expression while others may be selected to permit tissue specific expression. The cauliflower mosaic virus (CaMV) 35S RNA promoter is often used because it directs high levels of expression in most plant tissues (Walden and Wingender 1995).

6.1.2.1 Mechanism of *Agrobacterium* infection, T-DNA transfer and integration

Plant species differ greatly in their susceptibility to infection by *Agrobacterium tumefaciens* or *rhizogenes*. Even within a species, different cultivars or ecotypes may show different degrees of susceptibility. These differences have been noted in a variety of plant species. The subject matter has been reviewed (Gelvin 2000). Though environmental or physiological factors are attributed for these differences, genetic basis for susceptibility has recently been described in *Arabidopsis* (Nam *et al*. 1997).

Agrobacterium attaches to plant cells in a polar manner in a two-step process. The first step is likely mediated by a cell-associated acetylated, acidic capsular polysaccharide (Reuhs *et al*. 1997). The second step involves the elaboration of cellulose fibrils by the bacterium, which enmeshes large numbers of bacteria at the wound surface (Matthysse *et al*.1995).

The interaction between *Agrobacterum* sp. and plant involves a complex series of chemical signals communicated between the pathogen and the host cells. These signals include neutral and acidic sugars, phenolic compounds, opines (crown gall specific molecules synthesized by transformed plants), Vir (virulence) proteins and the T-DNA (Gelvin 2000). Baker *et al*. (1997) has described the chemical signaling in plant-microbe

interactions. The T-DNA transfer process initiates when *Agrobacterium* perceives certain phenolic compounds from wounded plant cells (Hooykass and Beijersbergen 1994) which serves as inducers or co-inducers of the bacterial *vir* genes. Phenolic chemicals such as acetosyringone and related compounds (Dye *et al*. 1997) are perceived via the VirA sensory proteins (Doty *et al*.1996). Most of the induced Vir proteins are directly involved in T-DNA processing from the Ti plasmid and the subsequent transfer of T-DNA from the bacterium to plant. Among them VirD2 and VirE2 contain plant active nuclear localization signal sequences (NLS) (Herrera-Estrella *et al*. 1990). VirD2 protein is directly involved in processing the T-DNA from the Ti plasmid. It nicks the Ti plasmid at 25-bp directly repeated sequences, called T-DNA borders that flank the T-DNA (Veluthambi *et al*. 1988). Thereafter, it strongly associates with 5' end of the resulting DNA molecule (Filichkin and Gelvin 1993) through tyrosin (Vogel and Das 1992). VirD2 contains two nuclear localization signal (NLS) sequences (Herrera-estrella *et al*. 1990) whereas VirE2 contains two separate bipartite nuclear localization signal (NLS) regions that can target linked reporter proteins to plant cell nuclei (Citovsky *et al*.1994).

Many plant species are still recalcitrant to *Agrobacterium* transformation. This recalcitrance does not result from a lack of T-DNA transfer or nuclear targeting, rather its integration into the genome of regenerable cells appears to be limiting. In the future, it may be possible to over express endogenous genes involved in the integration process or to introduce homologous genes form other species, and thereby effect higher rates of stable transformation (Gelvin 2000).

6.1.3 *Agrobacterium* -mediated transformation studies in papaya

Reports on transformation studies in papaya have been listed earlier (Table 1.12, Chapter 1). Pang and Sanford (1988) were the first to describe transformation of Sunrise Solo and Kopoho Solo using leaves, stem or petiole section as explants and inoculated with *Agrobacterium* strain GV3111 containing plasmid pTiB6S3; pMON200. The plasmid contained a transitionally improved chimeric NOS/NPTII/NOS gene for kanamycin resistance as a dominant selectable marker and tumor genes (tms/tmr) conditioning hormone independent growth. Although no transgenic plants were identified, a high rate (90%) of transformed callus was obtained.

Fitch *et al.* (1990) could transform the primary and secondary embryos of a highly embryogenic culture by insertion of a chimeric cP gene of the mild mutant starin HA 5-1 of PRV and GUS reporter gene in addition to NPTII gene of pGA482. The cauliflower mosaic virus 35S promoter was used to drive cP and GUS GENE while NPTII gene was driven by NOS (nopaline synthetase) promoter. Transformation of regenerated plants was confirmed by NPTII assays, PCR and Southern hybridizations of PCR amplified fragments (Fitch 1991). All the Ro transgenic plants tested by ELISA were negative for cP gene expression, including those tested positive for the presence of cP gene by PCR and Southern analysis.

In an another report, Fitch *et al*. (1993) used *Agrobacterium* strain C58-Z707 for transformation of hypocotyl explants from Kopoho cv. The plasmids pGA482GG or pGA 482GG/cpPRV-4 contained GUS and polylinker region in addition to kanamycin resistant gene NPTII. Four transformed lines were identified with two from each construct. However, they concluded that putative transgenic embryos appeared 6-9 months after co cultivation of embryogenic callus and somatic embryos with *A. tumefaciens* compared to four months when similar tissues were transformed via microprojectile bombardment (Fitch *et al.* 1990,1992).

Yang *et al.* (1996) could produce transformed lines of papaya from *Agrobacterium* infected petioles within 8 months of co cultivation. They used the plasmid pBII21 containing NPTII as a selective marker gene and GUS as a reporter gene. In the same year, Cabrera-Ponce *et al*. (1996) used *A. rhizogenes* to develop transgenic papaya plants of cv. 'Yellow large'. The strain used was LBA9402 having the vector pBI121 which contained a NPTII and GUS as selective and reporter gene. Although transformed papaya lines could be obtained by *A. rhizogenes* infection, the plants derived were not normal in morphology and also had poor growth. Recently Chen *et al.* (2001) have reported successful transformation of papaya with the viral replicase (RP) gene in cv. Tai-nong-2 *via A. tumefaciens*-mediated transformation. The RP gene was cloned in pRok vector and introduced in *A. tumefaciens* LBA 4404. With this construct 20 putative transgenic papaya were regenerated which were confirmed by PCR and Southern blot analyses

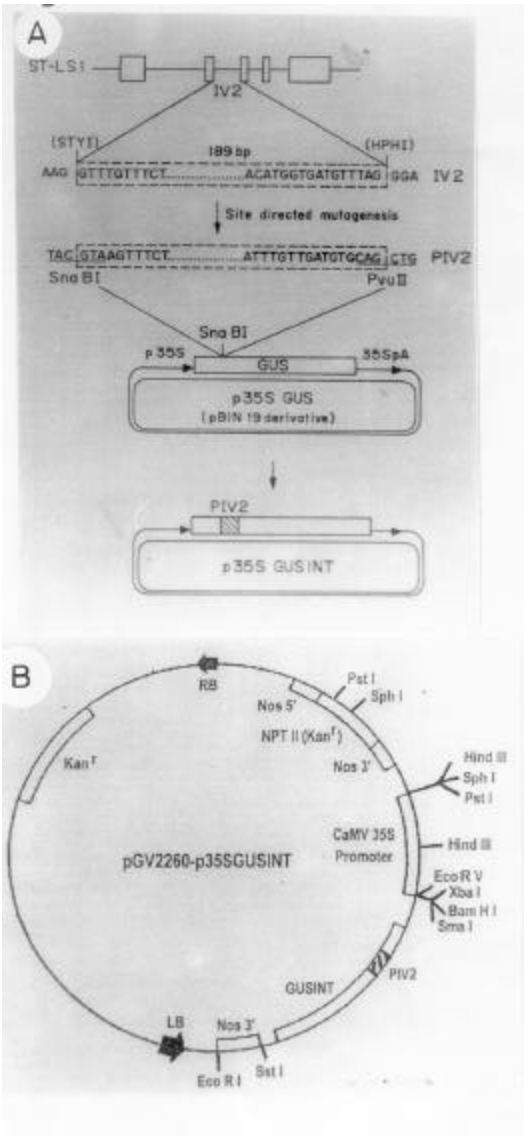


Fig. 6.1

A. *Agrobacterium* map of pGV2260-p35SGUSINT used in the present study. Source: Vancanneeyt *et al.* (1990).The ST-LS1 gene derived portable intron (IV2) is cloned in the β-glucuronidase (GUS)gene. The drawing also shows p35SGUS and its intron derivative p35SGUSINT

In our present study, initial efforts have been made to establish a genetic transformation protocol with chimeric marker genes for some of the important local culivars of papaya. Also, we have attempted to isolate coat protein gene and tried to clone it in a suitable vector pGEM3Zf (-). We have attempted this with an idea to introduce this Plasmid map of the *Agrobacterium* strain pGV2260-p35SGUSINT used in the experimentsgene to *Agrobacterium tumefaciens* strain for transformation in papaya. In this chapter, we present our entire work in two different sections. All experiments and results pertaining to *Agrobacterium* study has been discussed in section A followed by the description of isolation and cloning of CP gene in Section B.

6.2 Materials and methods

6.2.1 Plant material

Immature embryos were isolated from the seeds of cultivars Honey Dew and Co2 aseptically as described earlier in chapter 4. Before infection, the embryos were grown in MBG basal medium (MS salts+B5 viatmins supplemented with 2 mg/l glycine) with 3% sucrose and 2.0 mg/l 2,4-D for 5 days so as to allow the embryo axis to open up and the embryo axis becomes available for infection.

6.2.2 *Agrobacterium* strain

The Agrobacterium tumefaciens strain pGV2260 was used for the present study. The strain contains the plasmid p35SGUSINT which is a pBin19 derivative, carrying a chimeric NPTII gene and a GUS gene construct with a ST-LS1 gene derived intron under the control of CaMV 35S promoter (Vancanneyt et al.1990) (Fig.6.1). The strain was supplied by Prof. Deepak Pental (University of Delhi, North Campus, New Delhi, India).

6.2.3 Bacteriological media

Agrobacterium strain (pGV2260) was grown in YEB medium (Shaw 1988). The pH of YEB media was adjusted to 7.2 respectively. Compositions of the media is given in Table 6.1**.**

6.2.4 Growth media and conditions for *Agrobacterium*

The *Agrobacterium* strain was grown in YEB medium supplemented with antibiotics such as Kanamycin (100 mg/l), Streptomycin (50 mg/l) and Rifampicin (100 mg/l). The culture was grown at 200 rpm on a rotary shaker at 28 ^{0}C for 16 h. Aliquots of *Agrobacterium* culture at hourly intervals were pipetted out and the optical density of the

culture was measured at 600nm. The optical density was plotted against time to give the characteristic growth curve (Fig.6.2).

Table 6.1 Compositions of YEB media

Composition	*gm/l*
Bacto-yeast extract	***1***
Beef extract	***5***
Bacto-peptone	***5***
Sucrose	***5***
$MgSO_4$	***0.5***

YEB - (Yeast extract medium)

The culture after 18 h starts showing a decline in the optical density and thereby 18 hour grown culture was used for all the experiments.

6.2.5 Regeneration media

Explants were cultured either in a medium (A) containing Murashige and Skoog's (MS) basal medium supplemented with 2,4-D (2.0 mg/l) (herein after referred as M1) and 3% sucrose or (B) containing Murashige and Skoog's (MS) salts+Gamborg's (B5) vitamins + 2.0 mg/l glycine supplemented with TDZ (0.5 mg/l) and sucrose 3% (hereinafter referred as M2). Both the media were solidified with 0.75% agar (Hi-media, India).

6.2.5.1 Determination of lethal dose of kanamycin

The lethal dosage (LD_{50}) for kanamycin was determined by inoculating embryo axis explants on medium M1 and supplemented with various concentrations of kanamycin (50, 75, 100, 150 and 200 mg/l). The cultures were incubated for four weeks at 25±2 ^{0}C under a 16 h photoperiod at light intensity of 27 $\mu Em^{-2}s^{-1}$ provided by cool white fluorescent tubes.

6.2.5.2 Co-cultivation of explants

The embryo axes of the immature embryo explants were treated with *Agrobacterium tumefaciens* strain pGV2260. Over night grown *Agrobacterium* culture (18 h grown) was taken in 10 eppendorf tubes (1 ml in each) and spun at 5000 rpm for 10 min. at ambient temperature. The supernatant was discarded and the pellet obtained was resuspended in liquid medium of M1 or M2 (taking one ml in each eppendorf tube). Thus

a suspension was prepared and O.D was measured. O.D. was adjusted between 1 to 1.2 at 650 nm by adding the respective liquid M1 or M2 medium in the suspension. Explants were submerged in this *Agrobacterium* suspension (M1 or M2) for 5,10 and 20 and 30 min under sterile conditions, removed and blotted dry on a sterile filter paper and placed on two different media in 55 mm petridish either for callusing (media with 2,4-D referred as M1) or for multiple shoot formation (media with TDZ referred as M2). Six explants were cultured per dish. In this manner, around 500 explants per experiment were treated.

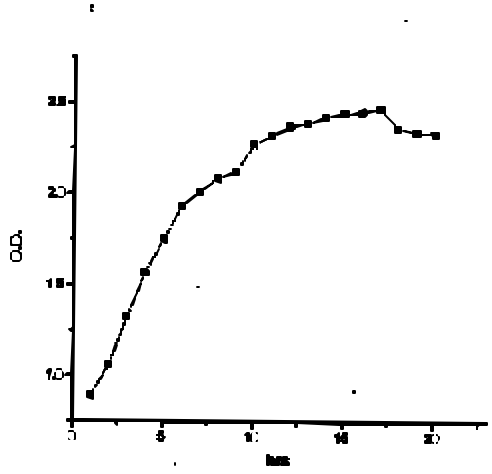


Fig. 6.2

A. Characteristics *Agrobacterium* growth curve plotted (OD_{600}) against time

All the treated explants were co-cultivated on semisolid medium M1 or M2 for 72 hours and incubated both in light and dark condition at 25 ^{0}C. These were then washed thrice with sterile double distilled water containing 500 mg/l of Claforan (Russel India Ltd), blotted dry again and cultured on semi-solid medium of M1 or M2 containing kanamycin (100 mg/l) + Claforan (500 mg/l). Explants not treated with *Agrobacterium* served as controls. After cocultivation all the cultures were incubated under 16 h photoperiod (light intensity of 27 $\mu Em^{-2}s^{-1}$) at 25±2 ^{0}C for 4 weeks.

6.2.5.3 Development of callus or embryo from embryo axis

Embryogenic callus developed from embryo axes on M1 medium containing kanamycin (100 mg/l) and claforan (500 mg/l) were transferred to freshly prepared medium. After four weeks of incubation, calli were again transferred to fresh medium having kanamycin (100 mg/l) and concentration of claforan halved at each subculture. The calli growing on the selection pressure were tested for GUS assay after four weeks of incubation.

On the other hand, explants cultured on medium containing TDZ with respective selection pressure, developed multiples shoots. These were further transferred to same medium containing kanamycin (100 mg/l) and claforan (500 mg/l) and incubated further as mentioned above.

6.2.6 GUS assay

Histochemical analysis was carried out to determine the β-glucoronidase activity in embryo axis and cotyledons after four weeks of treatment of *Agrobacterium tumefaciens*. Tissues were cut into small pieces and immersed in 1mM X-Gluc solution in microtiter multiwell plates and incubated overnight at 37 ^{0}C (Stomp 1992). Leaf sections from the chimeric shoots developed from embryo axis in medium supplemented with TDZ were also subjected to GUS assay after 5 weeks of incubation. The tissues were bleached in 100 % ethanol before observation. Details of the GUS solution are given in Table 6.2.

Table 6.2 Reagent mix for GUS assay.

Stock solution	**Final concentration**	**Reagent Mix ml/ml**

1.0 M NaPO4 buffer, pH 7.0	0.1M	100
0.25 M EDTA, pH 7.0	10 mM	40
0.005 M K-ferricyanide pH 7.0	0.5 mM	100
0.005 M K- ferrocyanide pH 7.0	0.5 mM	100
0.002 M X-glucuronide	1.0 mM	50
10 % triton X-100 (optional)	0.1 %	10
Subtotal		400
Distilled water		600
Final volume		1000

6.2.7 Histochemical test

After staining, the tissues were fixed by following the protocol developed by Meyerowitz (1987**).**

The procedure is as follows**:**

1. Previously stained tissue is placed in 100% ethanol sitting in a dry ice, 100% ethanol bath.
2. The ethanol immersed is transferred to a -70 ^{0}C freezer for 24-48 h (depending on tissue size) to allow the exchange of ethanol for water in the tissue
3. The tissue is slowly brought to room temperature with intervening incubations for several hours duration at -20 ^{0}C and 4 ^{0}C.
4. The tissue is placed into fresh 100% ethanol.
5. The tissue is incubated at room temperature for several hours and then given two 2 h passages in toluene. It is important that the tissue should sink in toluene before moving to wax indicating complete exchange of ethanol for water and toluene for ethanol.
6. The sample (tissue) is transferred to molten paraffin (65 ^{0}C) (Merck, E. Merck India Ltd., Bombay, India) and give 2 passages of 5-6 hours each

7. For sectioning, the tissue is embedded in paraffin wax The block was cut into 10 µm sections using a rotary microtome (Reichert -Jung 2050 supercut, Germany), mounted on a slide dewaxed in toluene and viewed under microscope (Stomp 1992).

6.2.8 Histomicrography

The sections were observed under a Stereoscan microscope and were photographed using a camera attached to a microscope (Docuval, Carl Zeiss Germany), on a Konica (Konica, Japan) colour film.

6.3 Results and discussion

The worldwide efforts to transform an ever increasing number of plant species with high efficiency has resulted in a number of promising gene transfer systems that continue to become more refined (Fisk and Dandekar 1993). Although, *Agrobacterium*-mediated transformation remains the method of choice, alternative methods such as electroporation and particle bombardment have also been developed in order to circumvent the poorly understood biological barriers, which prevent its application to certain plants (Fisk and Dandekar1993). In the present study, an attempt has been made to regenerate transgenic plants in papaya from embryo axes excised from immature seeds of two cultivars Honey Dew and Co2 and co-cultivated with *A. tumefaciens* strain carrying a plant expressive GUS gene interrupted by an intron. In this protocol, the GUSINT strain of *Agrobacterium* was used to avoid fake positives and selection pressure was applied immediately after the co-cultivation period.

Though papaya has been transformed by *Agrobacterium* and plants have been regenerated (Fitch *et al*. 1990,1993; Cabrera Ponce 1996), success has been limited to exotic varieties not cultivated in India.

Preferred explants for transformation studies include: immature embryos, proliferative shoot cultures and embryo axis derived from mature or immature seed for direct meristem transformation in dicotyledons (Christou 1996). Embryo axis has become the explant of choice for development of transgenic plants via Agrobacterium co-cultivation in chickpea (Kar et al. 1996; Krishnamurthy et al. 2000), peanut (Mckently et al. 1995) and peas (Davies et al. 1993). Use of embryo axis as an explant has several advantages: (1) due to its smaller size, it is amicable to both Agrobacterium as well as particle bombardment mediated transformation techniques, (2) the explant takes the least

time to develop into single shoot (10-15 days) compared to several months in case of plant regeneration via callus phase, (3) somaclonal variations can be avoided if callus phase can be bypassed, (4) and regeneration through embryo axis is genotype independent.

It is now widely accepted that the most suitable explants for transformation are those that require the least amount of time in tissue culture before and after the transformation step.

6.3.1 Lethal dosages of kanamycin

Explants on medium without kanamycin (controls) developed callus with embriods or shoots after 4 weeks of incubation. Embryo axes cultured on both M1 and M2 medium with kanamycin 50 and 75 mg/l showed initial callus or shoot development, however, these did not grow further and bleached subsequently. Survival of embryos were also observed on media supplemented with kanamycin 50 mg/l, however, concentrations of kanamycin at 100 mg/l and higher were lethal and inhibited the embryo growth completely (Fig. 6.3). Hence, kanamycin at 100 mg/l was taken as the selection pressure in all the experiments conducted with *Agrobacterium tumefaciens*.

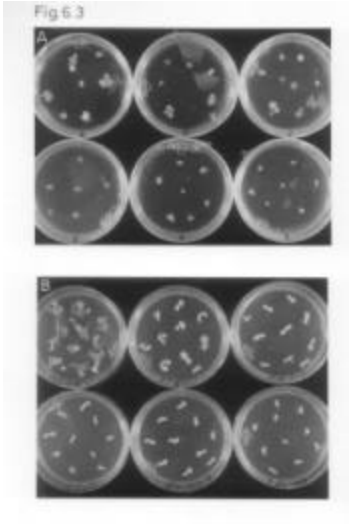


Fig. 6.3

A. Determination of lethal dose (LD_{50}) of kanamycin in papaya explants: a- control (M1), b-50 mg/l, c-75 mg/l, d-100 mg/l, e-150 mg/l, f-200 mg/l (M1-MS basal medium+2.0 mg/l 2,4-D)

B. Determination of lethal dose (LD_{50}) of kanamycin in papaya explants: a- control (M2), b-50 mg/l, c-75 mg/l, d-100 mg/l, e-150 mg/l, f-200 mg/l (M2-MBG medium+0.5mg/l TDZ)

6.3.2 Co-cultivation

Present section describes the results obtained from cv. Honey Dew as in our initial experiments with cv. Co-2, we have not achieved any positive results with *Agrobacterium* treatment and when cultured on selection medium containing kanamycin (100 mg/l). GUS activity in any of the tested explants could not be detected (500 explants were tested). However, a few embryos survived on selection medium after four weeks of culture only in cv. Honey Dew after *Agrobacterium* treatment and were found to be GUS positive. Hence, further experiments were carried out with this cv. to optimize the duration and mode of treatment of explants (5, 10, 20, 30 minutes etc.) with *Agrobacterium* suspension. The treated explants were co-cultivated with the *Agrobacterium* strain in both light and dark condition for 72 hours either in media M1 or M2 to determine the infection rate. We have observed the bacterial overgrowth in many of the explants cultured on either M1 or M2 when they were initially treated for a period of 30 mints (Fig.6.4A). Even after repeated washing and simultaneous inoculation on fresh medium containing antibiotics did not help to remove such overgrowth of bacteria. Earlier Cousins *et al*. (1991) has reported that frequency of transformation was largely influenced by the strain at use, method and period of co-cultivation and culture condition.

At the end of the co-cultivation period, we have cultured all the explants in medium containing antibiotic cefotaxime as it will specifically inhibit bacterial growth and a selective agent (kanamycin in the present study) that will select those cells receiving and expressing the gene transfer cassette (Hinchee *et al*. 1994).

6.3.3 Growth of treated embryos

A varied number of explants survived and developed callus or shoots on respective medium (M1 or M2) (Fig. 6.4) containing kanamycin (100 mg/l) depending on the mode of treatment and after 4 weeks of culture, however their number decreased in subsequent transfer to fresh medium containing selection pressure. We have noted a difference in number of surviving embryos when incubated in either light or dark condition for 72 hours co cultivation. Explants treated for 5, 10 and 20 minutes and co

cultivated in M1 media and kept in dark for 72 hours resulted in a survival of 2, 1 and 1 embryos respectively after 12 weeks of incubation (Fig.6.5). Among the eight batches

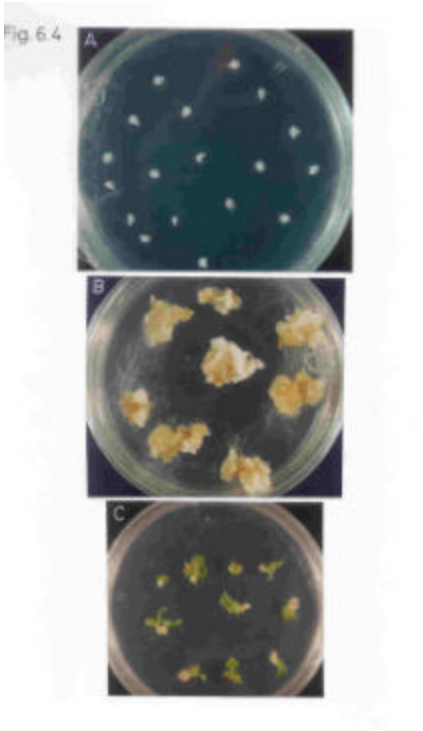


Fig. 6.4

A. Bacterial overgrowth observed after 72 h of cocultivation on the embryo explants, treated initially for 30 minutes with *Agrobacterium* suspension

B. Callus surviving (5 weeks old) on M1 medium supplemented with 100 mg/l kanamycin after cocultivation with *Agrobacterium* strain pGV2260-p35SGUSINT

C. Six weeks old shoots surviving on M2 medium supplemented with 100 mg/l kanamycin after treating with *Agrobacterium* strain pGV2260-p35SGUSINT

kept, explants that were co cultivated in the same media and in light condition for 72 h did not support growth of any embryo in the selective medium. Altogether 4 embryos survived (putatively transformed) upto a period of three months on selection pressure (Table 6.3). Untreated explants cultured on medium containing kanamycin (100 mg/l) did not survive.

In our experiment (500 explants were tested), explants treated with *Agrobactierum* suspension (M2) and co-cultivated in the same medium either in light and dark condition did not support the survival of any embryos after 12 weeks of culture on the selection medium (with 100 mg/l kanamycin). This apparently shows that dark incubation of the explants and media combination might have played a vital role in *Agrobacterium*-mediated transformation of explants in cv. Honey Dew.

6.3.4 Gus assay

We have not observed any endogenous GUS activity in control explants which were not co-cultivated with *Agrobacterium*. The maximum percentage (1.96) of embryo axes showed GUS activity when the explants were treated in *Agrobacterium* suspension (M1) for 5 minutes and cultured on semisolid medium (M1) for a period of 4 weeks. Treatment of embryo axes with *Agrobacterium* suspension (M1) for 5, 10 and 20 minutes and co-cultivated in dark resulted in 1.96, 1.28 and 0.81 % of explants showing GUS expression (Table 6.3). Transformed callus (Fig. 6.6) obtained from embryo axis showed intense blue color compared to non transformed callus (Fig.6.7).

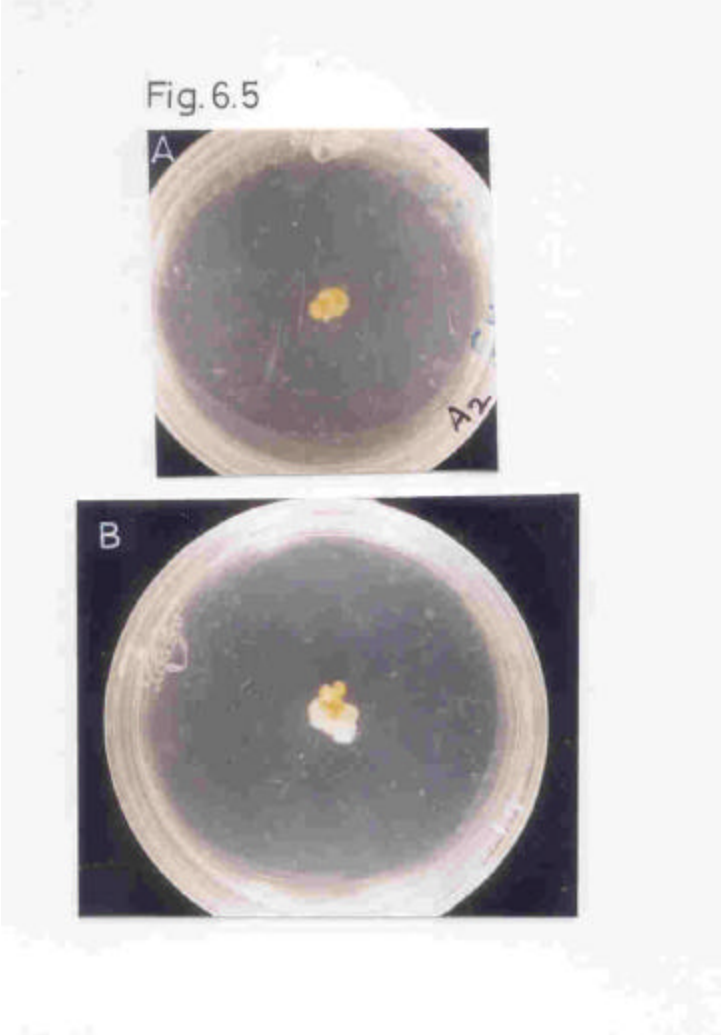


Fig. 6.5

A. Treated embryo explant (for 10 minutes in *Agrobacterium* suspension) surviving on the M1 medium supplemented with 100 mg/l kanamycin after 3 months of culture

B. Treated embryo explant (for 5 minutes in *Agrobacterium* suspension) surviving in the M1 medium supplemented with 100 mg/l kanamycin after 3 months of culture

In the present study, some callus samples excised from the embryos (putative transformants) surviving on selection pressure for 12 weeks of culture did not show any GUS activity.

In an earlier report by Cousins *et al*. (1991), it was stated that, not all the NPTII expressing plants showed GUS activity, presumably because of premature termination of the introduced T-DNA during the transfer process.

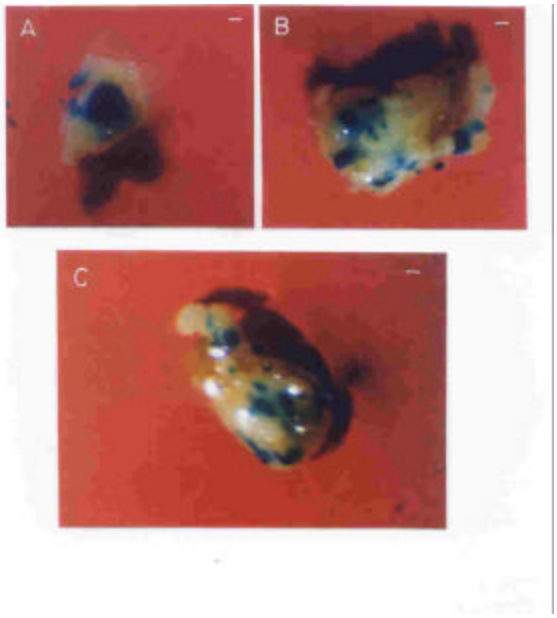


Fig. 6.6
GUS gene expression in callus tissue of papaya explants after 4 weeks of culture.
A. Callus derived from immature embryo explant treated for 5 minutes in *Agrobacterium* suspension.Bar=450 µm

B. Callus derived from immature embryo expalnt treated for 10 minutes in *Agrobacterium* suspension. Bar=450 µm

C. Callus derived from immature embryo explant treated for 20 minutes in *Agrobacterium* suspension.Bar=428 µm

Few explants survived in shoot multiplicatin medium (M2) and developed shoots, however, they were found GUS negative when tested for GUS assay. Remaining shoots were transferred to fresh medium containing kanamycin 100 mg/l for their further growth but all of them gradually died in selection medium.

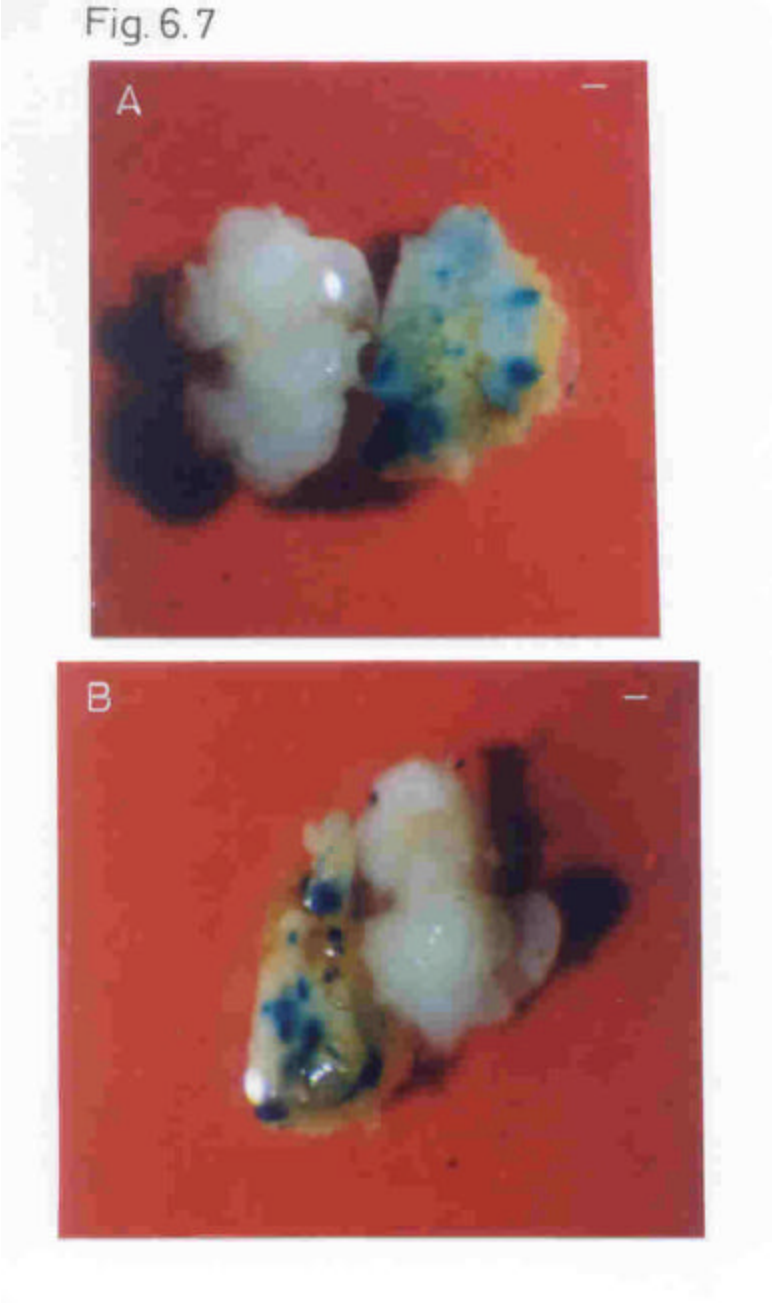


Fig. 6.7
Comparison of transformed and non transformed callus (blue colour indicates GUS gene expression)

A. Callus obtained from explants treated for 5 minutes in *Agrobacterium* suspension. Bar=444 µm

B. Callus obtained from explants treated for 10 minutes in *Agrobacterium* suspension.Bar=452 µm

Cleavage of 5-bromo, 4- chloro, 3-indolyl, β-D-glucoronide (X-gluc) by the enzyme β-glucoronidase produces the final insoluble blue colour precipitate dichlorodibromoindigo (ClBr-indigo) (Fig. 6.8). It is readily detectable at low concentrations, final cleavage product is insoluble in water; reaction proceeds to an

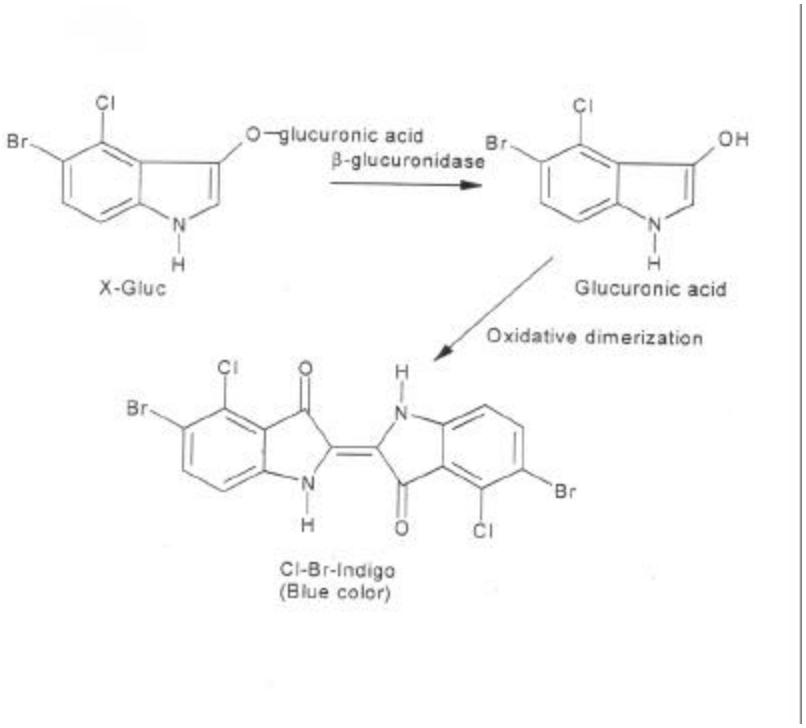


Fig. 6.8

A. Cleavage of 5-bromo, 3-indolyl, β-D-glucuronide by the enzyme β-glucuronidase into dichloro-dibromo-indigo (Cl-Br-indigo)

insoluble intermediate which on oxidative dimerization gives intense blue colour, and this product allows precise cellular localization and little loss of enzyme product on tissue processing. Cellular localization of the GUS was observed and confirmed by histological studies (Fig. 6.9)

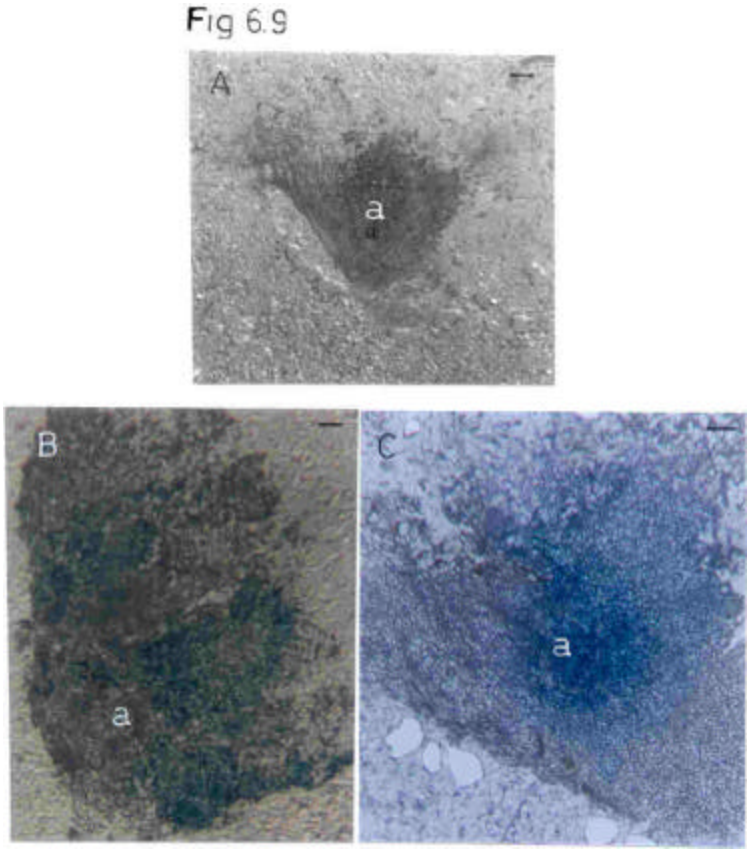


Fig. 6.9

A. L.S of transformed callus obtained from explants treated in Agrobacterium *suspension for 5 minutes. (a-Deep blue colour indicates the cellular localization of the GUS). Bar=620 mm*

B. L.S of transformed callus obtained from explants treated in *Agrobacterium* suspension for 10 minutes (a-Deep blue colour indicates the cellular localization of the GUS). Bar=270 µm

C. L.S of transformed callus obtained from explants treated in *Agrobacterium* suspension for 20 minutes (a-Deep blue colour indicates the cellular localization of the GUS). Bar=312 µm

In the present study, use of Agrobacterium strain containing a portable intron in the β-glucuronidase (GUS) gene has ruled out the possibility of false GUS activity (blue colour) in histochemical test of the explants due to bacterial contamination. The introduction of the portable intron into the GUS gene leads to nearly complete repression of its expression in Agrobacteria because of the absence of the eukaryotic splicing apparatus in prokaryotes. Use of such construct avoids the confusion of GUS gene expression in inoculated explants (Vancanneyt et al. 1990).

Table 6.3 b **-glucuronidase expression in embryo axis of papaya (*Carica papaya* L.) cv. Honey Dew cultured in M1 medium after inoculation with *Agrobacterium tumefaciens* strain GV2260 / p35SGUSINT**

No. of explants treated	Treatment period (min)	Co cultivation condition	No. of explants tested for GUS assays after 4 week of culture	No. of explants found GUS +ve	% of explants showing GUS expression	No. of embryos formed after 4 weeks of culture
510	30	**Dark**	70	0	0.00	0
600	20	Do	123	1	0.81	0
510	10	Do	78	1	1.28	0
600	5	Do	102	2	1.96	0
200	30	**Light**	23	0	0	0
500	20	Do	58	0	0	0
310	10	Do	56	0	0	0
300	5	Do	48	0	0	0

6.4 Conclusion

In our present study, we have noted that frequency of transformation in papaya cv. Honey Dew is largely influenced by the period of treatment of Agrobacterium

tumefaciens strain harboring plasmid p35SGUSINT and also by the media and the co cultivation culture condition (light and dark incubation). Treatment of explants in Agrobacterium suspension (M1) for 5 minutes followed by dark incubation and cultured in semi-solid medium seems to be the ideal condition of treatment for affecting transformation in embryo axes. In our study, co cultivation of the treated explants in dark condition for a period of 72 hours could produce more number of putative transformants, whereas explants co-cultivated in light (27 $\mu Em^{-2}s^{-1}$) did not favour the Agrobacterium infection of explants. The integration of GUS genes in callus tissue has been confirmed by histological analysis. Intense GUS activity was observed in callus tissue and embryo axis. The present study on transformation of papaya could be of immense importance as report on genetic transformation studies on Indian cultivars is presently not available.

Section B

6.5 Introduction

Papaya Ringspot Virus (PRSV) belongs to the group Potyvirus, which is in turn the largest group of viruses that causes significant loses in agricultural, horticultural, pastural and ornamental crops (Sukhla *et al*. 1991, Riechmann *et al*. 1992). The potyvirus group is named after its type member Potato Virus Y (PVY) and is the largest of the 34 known plant virus groups and families constituting about 30% of all known plant viruses.

6.5.1 The physical characteristics of potyvirus

The morphological characteristics of the virions are reported to be flexous and rod shaped. The dimensions of a potyvirus particle range from 680-900 nm in length and 11-15 nm in width (Sukhla *et al*. 1991,Riechmann *et al*. 1992). Each virion is made up of 2000 units of a single structured protein surrounding one molecule of single stranded RNA that is on an average 10,000 nucleotides long and of messanger polarity. Definitive potyviruses are transmitted in a non persistent manner by many aphid species, while some of the poty viruses have fungus, mite or whitefly vectors (Sukhla *et al*. 1991). All potyviruses examined so far are reported to induce the characteristics pinwheel or scroll shaped inclusion bodies in the cytoplasm of the infected cells (Edwardson 1974).

6.5.1.1The genome

The potyvirus genome comprising of a ss RNA has a protein (VPg) covalently attached to its 5' end and a single open reading frame (ORF) coding for a **large**

polyprotein precursor and a poly (A) tail at the 3' end. This ORF is flanked by two non-coding regions (NCR) at the 5' and the 3' ends (Riechmann *et al.* 1992, Hari *et al.* 1981, Hari 1981).

6.5.1.2 The 5' non-coding region

The length of the potyviral 5' NCR ranges between 144 to 205 nucleotides (Sukhla *et al.* 1991, Riechmann *et al.* 1992). They are rich in A and have few G (Riechmann *et al.* 1992). Alignment of the 5' NCR of a few viruses like Plum pox virus (PPV), Tobacco etch Virus (TEV), Tobacco vein mottling virus (TVMV) and PVY have reavealed a marked differences in their sequence except for some conserved nucleotide blocks and is not accompanied by the conservation of their predicted secondary structures (Turpen 1989). There is a highly conserved region of 12 nucleotides, TCAACACAACAT, which is termed as the potybox, starting at position 13 in PVY and TVMV and at position 14 in PPV and TEV . The conservation of these sequences suggests that they could play an importamt role in the viral life cycle for example in the process of encapsidation, translation or replication (Sukhla *et al.* 1991).

The PVY strain N (PVY^{N}) RNA genome has a 5' NCR of 185 bases (Robagalia *et al.* 1989). There are two other reading frames that are frequently punctuated by termination codons and are therefore not likely to code for proteins of significant size. The first AUG codon at position 185 is likely to be a initiator codon (Robagalia *et al.* 1989) since it is in a context (UCAAUGGC) similar to the consensus sequence for translation initiation in plants (AACAAUGGC) (Lutchke *et al.* 1987). Strains of PVY have a penta nucleotide motif that repeats 3-5 times in the region and it appears only once in the same region of some other potyviruses except in case of TVMV where it occurs thrice. This penta nucleotide consrved motif is not found at the 5' end of the complimentary strand which is in agreement with other conserved sequence elements and could have a role in the PVY life cycle (Thole *et al.* 1993).

6.5.1.3 The 3' non-coding region

The 3' non-coding region of different potyviruses has been described as heterogenous in size, sequence and predicted secondary structures. The Length varies from 147-499 nucleotides (Ward and Sukhla 1991). Distinct potyviruses show a low sequence identity in the 3' NCR (up to 30 to 53%) as compared to that between strains of

individual viruses (Frenkel *et al.*1989). This region consists of pairs of direct repeats and is capable of forming secondary structures (Dougherty *et al.*1985). Features seen common to all of them are the presence of AU -rich segments and that each of them can be predicted to fold into stable secondary structures (Turpen 1989). However, their predicted secondary structures appear to vary from virus to virus and their biological significance in potyviruses remain unclear (Hay *et al.*1989, Turpen 1989). In case of papaya ringspot virus (PRSV) strains W and P, no obvious common features of secondary structures were observed although extensive secondary structures were predicted with a wide range of free energies (Quemada *et al.* 1990). PRSV-W infects both papaya and cucurbits while the latter type (P) infects cucurbits but not papaya. In fact, PRSV-W causes major damage to cucurbits and was previously referred to as watermelon mosaic virus I. Both types cause local lesions on *Chenopodium quinoa* and *C. amaranticolor.*

6.5.1.4 The coat protein

Coat protein is the most characterised gene product of potyvirus. Coat protein (CP) monomers are reported to range in size from 30-45 kDa. Heterogeneity in apparent CP size is a common feature of potyvirus owing to degradation during purification and /or storage (Dougherty *et al.* 1988). These degraded forms moves faster on SDS - polyacrylamide gels as specific bands (Huttings and Mosch 1974, Heibert and McDonald 1976). From biochemical and genetic analysis, however it is clear that each potyvirus contains a single type of capsid protein monomer (Allison *et al.* 1985).

N-termini of CP of distinct potyviruses vary considerably in length and sequence whereas the C terminal two thirds of the protein are highly similar as revealed by sequence comparisons and biochemical analysis (Ward and Sukhla 1991, Sukhla and Ward 1988). The significant properties of the N-terminus (Sukhla and Ward 1989, Sukhla *et al.* 1988,1989) are:

i) It is the only large region in the entire CP that is unique to a potyvirus

ii) It is immunodominant

iii) It contains virus specific epitopes

Mild proteolysis by trypsin of purified potyvirus particle revealed that N- and C

terminal regions of the CP are exposed on the particle surface (Sukhla *et al.* 1988) The surface exposed N- terminal region can vary in length from 30 to 95 amino acids, depending on the virus, whereas the length of the surface exposed C- terminus is only 18-20 amino acids in different potyviruses (Ward and Sukla 1991, Sukhla *et al.* 1988). Removal of the N- and C- termini by proteolysis leaves a fully assembled virus partcle composed of coat protein cores consisting of 215 to 227 amino acids (Sukhla *et al.* 1991). These core particles appear indistinguishable from untreated native partcles by electron microscopy and are still infectious suggesting that N- and C- termini are not required in partcle assembly or for infectivity during mechanical inoculation (Sukhla *et al.*1988, Jagadish *et al.* 1991).

6.5.1.4.1 The role of coat protein in potyviral transport

The involvent of CP in the potyvirus movement was revealed by the analyisi of TEV (Dolja *et al.* 1994). In addition to virion assembly, CP plays significant role in cell to cell movement and long distance transport. Two possibilities are envisaged. Firstly, cell to cell movement may require the assembly and translocation of virions between host cells. Alternatively, a non virion ribonucleo protein may be the transported form (Dolja *et al.* 1994), analogous to that proposed for several other viruses that donot require virus assembly prior to movement (Deom *et al.* 1992). Potyviruses differ in that CP may participate in the function of the ribo nucleoprotein complex or may interact with the host or other viral factors to potentiate transport in a manner comparable to a movement protein (Dolja *et al.* 1994). Mutations in the N- terminus did not affect virion assembly but partially inhibited cell to cell movement indicating that CP has other roles to play in translocation, besides assembly (Dolja *et al.* 1994). These mutations inhibited systemic translocation through the vascular tissues which was rescued by complementation with the transgenic wild type CP thereby confirming the role of CP in long distance translocation (Dolja *et al.* 1994). Mutations in the CP core domain debilitate the interactions necessary for en capsidation and possibly affect protein-protein or protein-RNA interactions involved in the formation of a non virion transport complex. RNA sequences involved in the assembly of virions are yet to be identified for their role in movement (Dolja *et al.* 1994). Factors interacting with CP for virus movement are yet to be identified. CP is probably the only viral protein necessary in potyviruses. However, a

movement function may be performed by one or more structural proteins that are involved in other essential functions during replication and infection (Dolja *et al.* 1995).

6.5.1.4.2 The role of coat protein and helper component in vector pathogen interactions

Successful transmission of potyviruses by their aphid vectors depends upon the interaction of two viral encoded proteins, CP and HC (Helper component) (Pirone 1991). Enzymatic removal of the N-terminal domain of the CP results in the loss of aphid transmissibility (Salomon 1989). A DAG (aspartic acid-alanine -glycine) conserved motif in the N-terminal region is said to play a role in aphid transmissibility (Harrison and Robinson 1988). Deletions of DAG motif abolished aphid transmission in TVMV (Atreya *et al.* 1991). Changes in the second or third amino acid in the DAG motif is a consistent feature of non transmissible potyvirus isolates. Amino acids downstream of this motif were also postulated to have an effect on aphid transmissibility (Atreya *et al.* 1991). This motif was suggested to be expanded to DAGX although the fourth amino acid is not highly conserved (Atreya *et al.* 1995).

It has been suggested that the helper component (HC) functions either by acting as a bridge binding to the DAG motif in the CP and to retention sites in the aphid mouth parts or by indirectly mediating an interaction between DAG and the aphid (Pirone 1991). The HC of non transmissible isolate of TEV was found to be functional in the transmission of another isolate implying that it does not recognize the CP of the former. HC of PVY can assist the transmission of non transmissible isolate of TEV (Pirone and Thornbury 1983); adaptation of CP to its own HC is lost. It has been proposed that more than one site in HC may be functionally related to aphid transmissibility (Huet *et al.* 1994). Cleavage at DAGX sites by a trypsin like enzyme might release the particles from their retention sites in the aphid feeding apparatus so that they could be egested and act as inoculum during subsequent exploratory probes by the aphids (Harrison and Robinson 1988). Such cleavages are not known to destroy infectivity (Heibert *et al.* 1984).

When potyviruses are subcultures several times without the use of aphid vectors they tend to lose their aphid transmissibility (Simons 1976). Presumably because aphid non-transmissible variants outgrow their transmissible progenitors. In some cases the key change is in the virus particle protein but in others it is in the helper component (Granier

et al. 1993). Both theses proteins must therefore be conserved and retain their mutual adaptation if they are to function in transmission and the virus is to maintain its biological fitness (Harrison and Robinson 1988).

6.5.2 Papaya ringspot virus

Papaya ringspot is one of the most destructive diseases of papaya and occurs in nearly every region where papaya is grown except for Africa. It is particularly severe in areas of Thailand, Taiwan, the Philippines, the southern region of The People's Republic of China and India. The host range of PRSV is limited to plants in the families Caricaceae, Cucurbitaceae, and Chenopodiaceae. Symptoms induced by different isolates of PRSV-p may vary in intensity, but dark green, often slightly sunken, rings are diagnostic. The number of rings on fruits can be variable, and the rings become less distinct as the fruit matures and yellows. Fruits often show uneven bumps, especially those fruits that develop after a tree is infected.

Other key symptoms are intense yellow mosaic on leaf lamina and numerous "oily" streaks on petioles. The leaf canopy becomes smaller as the disease progresses due to the development of smaller leaves and stunting of the plant. Fruit yield and brix levels are markedly lower than fruit from healthy plants. Leaf and fruit symptoms are most intense during the cool season. Leaves often develop a shoestring appearance caused by the extreme reduction of leaf lamina similar to that caused by broad mites.

Papaya trees of all ages are susceptible and generally will show symptoms two to three weeks after inoculation. Trees infected at a very young stage never produce fruits but rarely dies because of the disease. There are, however, some isolates in Taiwan, which cause wilting, and sometimes death of young trees.

Papaya ringspot virus can be rapidly spread by several aphid species in a non persistent manner. Though many cucurbits are susceptible to PRSV-P, they do not serve as an important alternate host. Instead, the dominant strain in cucurbits is PRSV-W. Therefore, the spread of the virus (PRSV-P) into and within an orchard is primarily from papaya to papaya. There is no evidence that PRSV can be transmitted through seeds from infected papaya or cucurbits.

The development of the disease in an orchard follows the general pattern of viruses that are spread by aphids in a non-persistent manner. The amount of primary

infection increases as the distance from infected papaya trees decrease. Secondary infection spreads rapidly and an orchard can become totally infected in three to four months. This situation occurs in young orchards located close to infected plants and during periods when populations of winged aphid flights are high.

PRSV is a member of the potyvirus genus in the potyviridae. Papaya ringspot viruses are typically long flexuous rod-shaped particles filamentous and not enveloped, about 800-900 nm long and 12 nm wide. Virions contain 5.5% nucleic acid; 94.5% protein and is the longest encountered among the sequenced potyviruses with a total genome size of 12 kb. PRSV RNA which is single stranded and linear encodes a large polyprotein of 381 kDa (Yeh and Gonsalves 1985, Yeh *et al*. 1992) which is processed into 8 or 9 final products via three virus-encoded proteinases (P1, HC-Pro and Nia). Like other potyviruses the proposed genetic organization pf PRSV RNA is VPg-5' leader-P1 (63K)- HC Pro-p3 (46k)-CI-P5 (6K)-NIa-NIb-CP-3'noncoding region-poly (A) tract (Yeh et al 1992). The P1 protein of PRSV is the most variable protein among potyviruses and it has a size of 63 kDa which is 18-34 kDa larger than those of the other potyviruses (Yeh *et al*. 1992). Virions are found in all parts of the host plant; in cytoplasm and in cell vacuoles. Inclusions present in infected cells are amorphous X-bodies and pinwheels (i.e. scrolls (Eddwardson's type 1inclusions)); they don't contain virions. These are transmitted by numerous species of aphids in a nonpersistent manner. Papaya ringspot virus is grouped into two types, PRSV-P and PRSV-W. PRSV - type P infects papaya and cucurbits, but watermelon mosaic virus 1 (PRSV-W) infects cucurbits, but not papaya. The two types are very closely related, except for the inability of type W to infect papaya. Analysis of the coat protein genes of isolates from Australia, Thailand and the U.S.A. indicates that, in each country, papaya ringspot - P isolates have evolved independently through mutation from local watermelon mosaic virus 1 isolates (Bateson *et al*.1994). These two strains share 97.7% nucleotide sequence similarity and 97.2% amino acid similarity. Infections of crop plants by viruses can lead to reduced yield lowered product quality and sometimes to complete crop failure. Chemical methods that are available to control other types of plant pathogens cannot be used to control plant viruses since they are intracellular, obligate parasites. Several approaches have been taken to introduce virus resistance into desired crop plants like, cross protection, generating transgenic

plants which produce components of the virus that confer cross protection without causing viral disease (processes involving virus coat protein, replicase, movement protein, transmission protein, disease attenuation with satellite RNA defective interfering RNA and antisence RNA). Coat protein (CP) mediated resistance has been the most consistent and effective of these (Beachy *et al*. 1990). Plants transformed with CP gene of a pathogenic virus may in some cases be virtually immune to infection by the same or a closely related virus or may show delayed or reduced symptom expression. CP mediated resistance has been successful in test involving different groups of plant viruses like potyviruses (Lawson *et al*.1990) to which PRSV belongs and the potex virus group (Hoekema *et al*. 1989, Lawson *et al*. 1990) to which papaya mosaic virus (PMV) belongs. Coat protein mediated protection is found to be very successful for viruses with single stranded RNA genome. Hence, there is reason to believe that this approach will be useful in improving virus resistance in papaya.

As with conventional cross protection (Wang *et al*. 1987, Yeh *et al*. 1988) transformation of papaya with PRSV CP gene to produce genetically engineered virus resistance will preserve the genotype and horticultural merits of the original cultivar. In addition, there are a number of important ways in which the transgenic plants may prove to be superior to cross protected plants: i) stable incorporation of the CP gene in a chromosome of the transformed plant will result in Mendelian inheritance of virus resistance in the seedling progeny, eliminating the need to inoculate each generation with a mild protective viral strain, ii) expression of the CP gene in every cell of the transformed plant should reduce the frequency of the breakdown in resistance such as may occur in conventionally cross protected plants due to poor systemic spread of the mild resistance iii) The absence of a replicating albeit mild, virus in the genetically engineered plants mean that (a) there should be no disease symptoms or yield reductions, and (b) there will be no possibility of a mild virus escaping to infect other crops.

Two transgenic cultivars, Rainbow and SunUp, that are resistant to PRSV in Hawaii were recently commercialized (Gonsalves 1998, Manshardt 1999). Sun Up was derived from transgenic papaya line 55-1 (Tennent *et al*. 2001) a mild mutant of PRSV HA (Yeh and Gonsalves 1984). Rainbow is a hybrid of SunUp and the non-transgenic cultivar 'Kopoho'. It is therefore hemizygous for the CP gene (Mansherdt 1999). Tennant

et al. (1994, 2001) reported that Rainbow and hemizygous plants of line 55-1 are resistant to PRSV isolates from Hawaii that share at least 97% nt identity to the CP transgene but are susceptible to isolates from outside Hawaii that have 89-94% identity to the transgene. In contrast, Sun Up is resistant to a number of isolates from outside Hawaii.

6.6 Materials and methods

Leaves of papaya cultivar Honey Dew and Co-2 showing green islands between veins and reduced size were collected and used for total RNA isolation.

6.6.1 Nucleic acid

Elimination of ribonuclease from the experimental system

To obtain good preparation of RNA from leaf tissue it was necessary to minimize the activity of ribonucleases liberated during cell lysis (Sambrook *et al*. 1989; Blumberg 1987). The following precautions were taken to minimize RNase contamination:

Glassware: All glassware was treated with 0.1% diethylpyrocarbonate (DEPC) overnight and then autoclaved after draining off the solution. This was followed by baking at 300 ^{0}C for 4 hours.

Plasticware: All plasticwares were siliconized to render their surfaces hydrophobic. This was followed by treatment with 0.1% DEPC overnight and subsequent autoclaving.

Solutions: All solutions were prepared in deionized water 0.1% DEPC treated and autoclaved. It was imperative to post treatment denature DEPC by autoclaving since it modifies the purine residues in RNA and -NH_2 moeities in amino compounds such as Tris. All solutions were stored in DEPC treated, autoclaved and baked containers.

Some plasticwares such as electrophoresis units etc were found to react to DEPC. These were treated with 3% hydrogen peroxide overnight and subsequently rinsed extensively with DEPC treated deionized water (Sambrook *et al*. 1989).

In addition to the above precaution chaotrpic agents such as guanidium salts and reducing agents such as 2 -mercaptathanol which inactivate RNases (Chirgwin *et al*. 1979; MacDonald 1987) were used for the isolation of RNA. The method of Frenkel *et al*. (1989) with some modification was followed for RNA preparation.

6.6.2 Isolation of RNA from leaf tissues

Solutions needed

Grinding Buffer (GB)

50 mM Tris HCL, pH 8.0

7 mM EDTA , pH 8.0

1 mM $MgCl_2$

5 mM β- mercaptoethanol

0.1% BSA

Lysis Buffer (LB)

50 mM sodium acetate, pH 5.0

1 mM EDTA , pH 8.0

0.5% SDS

Other solution and reagent

Distilled phenol containing 0.1% 8-hydroxyquinoline and saturated with DEPC treated water

Chloroform: isoamylalchohol in the ratio of 24:1.

Process involved

Total of 15g young infected leaf tissue was cut into small pieces without the midrib. These pieces were transferred to ice cold mortar and pestle and ground to a fine powder in presence of liquid nitrogen. Grinding buffer (GB) was added to the fine tissue powder, allowed to thaw and filtered through muslin cloth. The filtrate (7.5 ml in each tube) was distributed in COREX glass tubes kept on ice. About 2.5 ml of lysis buffer was added to each tube, mixed vigorously and incubated for 10 minutes at room temperature. After that an equal volume of phenol was added and the mixture was centrifuged at 4^0C 10, 000 rpm for 10 minutes. The supernatant was collected and an equal volume of chloroform: IAA added and centrifugation was repeated as above.. After centrifugation the aqueous phase was collected and to it 1/10 volume of 3M NaOAC (sodium acetate) and equal volume of isopropanol was added. The samples were incubated at -20 ^{0}C overnight. The sample was centrifuged at 4^0C at 8, 000 rpm for 10 minutes. The precipitate was resuspended in 200 µl 70% ethanol by vortexing and recentrifuged at 4 ^{0}C at 8, 000 rpm for 10 minutes. The pellet was air dried, dissolved in 500 µl of DEPC water and 1/2 volume (250 µl) of 8M lithium chloride and incubated at -20 ^{0}C overnight. The samples was centrifuged for 15 minutes at 4^0C at 10, 000 rpm. Thereafter, the pellet was washed with absolute alcohol and again centrifuged for 10 minutes at 4^0C at 10, 000 rpm.

The precipitate obtained was dried, dissolved in minimum amount of water and run on formaldehyde agarose gel for visualizing the isolated total RNA.

6.6.3. Separation of RNA in denaturing agarose gels

RNA molecules do not have hydrodynamically equivalent conformations in aquoeus solutions and therefore several methods were developed in which molecular weights of RNA could be determined by gel electrophoresis under denaturing conditions (Lehrach *et al*. 1977). Formaldehyde (HCHO) the first denaturant used for the electrophoretic analysis of some RNAs without appreciable degradation also required the presence of formamide (Lehrach *et al*. 1977).

Reagents

Reagent A (gel electrophoresis buffer-10X stock)

0.5 M MOPS pH 7.0

0.01 M EDTA pH 7.5

This solution was kept in the dark at 4^0C.

Reagent B

294 µl reagent A+ 706µl distilled water

Reagent C

89 µl HCHO (37%) + 706 µl formamide

Reagent D (gel loading buffer)

322 µl reagent B

5 mg xylene cyanol

400 mg sucrose

178µl HCHO (37%)

500µl formamide

Reagent E

0.5 mg/ml ethidium bromide

Gel preparation

1% agarose in 1X MOPS/EDTA

0.5 g agarose

5 ml Reagent A

36 ml distilled water

The above was heated to mix the agarose and cooled to 60^0C. 9 ml of 37% HCHO was added making the final concentration of HCHO to 2.2 M. This was mixed well and poured into a gel casting mould.

Sample preparation

The RNA sample was dried under vacuum and dissolved sequentially in the following manner:

2.2 µl Reagent B

4.8µl Reagent C

1.0µl Reagent E

The above was heated to 70^0C for 10 min and quenched on ice. Reagent E (ethidium bromide) was added for better staining of low amount of RNA without any significant background (Gong 1992). 1.5 µl of Reagent D was added, mixed well and sample loaded on the gel.

6.6.3.1 Electrophoresis

Electrophoresis was carried out in 1X MOPS/EDTA. Pre-electrophoresis was done at 60V for 30 min. After loading the samples electrophoresis was carried out at 60V for 1 hour and then at 100V for 1-2 hours. Halfway through the process the electrophoresis buffer was remixed thoroughly and poured back into the electrophoresis unit. Any ionic imbalance at the electrodes was thereby taken care of. The gel was photographed over an ultraviolet transilluminator fitted with polariod camera or photographed on GEL DOC unit (Amersham pharmacia biotech).

6.6.4 Preparation of poly $(A)^+$ RNA

Preparation of poly $(A)^+$ RNA was done according to Ausubel (1993).

Solutions

DEPC water

Oligo (dT) cellulose

0.1M NaOH

10 M lithium chloride

2mM EDTA /0.1% SDS

3M sodium acetate

RNase free TE buffer

Silanized column

Silanized centrifuge tubes

5M NaoH

Poly A loading buffer

0.5M lithium chloride

10 mM Tris HCl (pH-8.0)

1mM EDTA (pH-8.0)

0.1% SDS

Middle wash buffer

0.15 M lithium chloride

10 mM Tris HCl (pH-8.0)

0.1% SDS

A silanized column of 2 ml capacity was washed with 10 ml of 5 M NaOH. Slurry of 0.5 g dry Oligo (dT) cellulose powder with 1 ml of 0.1 M NaOH was made and poured inside the tube. The tube was washed with 10 ml water. The column was equilibrated with 10-20 ml loading buffer. At the end of wash, pH at the output maintained was 7.5. About 2 mg total RNA was heated to 70^0C for 10 minutes. 0.5 M lithium chloride ($LiCl_2$) was added from a concentration of 10M stock solution. The RNA solution was then passed through the oligo (dT) column. The column was washed with 1 ml Poly (A) loading buffer. The elute was then passed trice through the column. The column was washed with 2 ml middle wash buffer. The RNA was eluted freshly in a tube with 2 ml of 2 mM EDTA and 0.1%of SDS. The whole process was repeated thrice. RNA was precipitated by addition of 0.3 M sodium acetate, 2.5 vol ethanol and overnight incubation -20 ^{0}C. The sample was then spun at 5000 rpm for 30 minutes at 4 ^{0}C. Ethanol was removed and the pellets were air dried and resuspended in 150 µl TE buffer. RNA quality is then checked by heating 5 µl at 70^0C for 5 minutes and analysing on 1% gel (agarose).

6.6.5 Amplification by RT Polymerase Chain Reaction

The polymerase chain reaction is used to amplify a segment of DNA that lies between two regions of a known sequence (Sambrook *et al*. 1989). Two oligo nucleotides are used as primers for a series of synthetic reactions that are catalyzed by a thermostable DNA polymerase. These oligo nucleotides typically have different sequences that lie on opposite strands of the template DNA and flank the segment of DNA that is to be amplified. The template is first denatured by heating in the presence of alarge molar excess of each of the two oligo nucleotides and the four dNTPs. The reaction mixture is then allowed to cool down to a temperature that facilitates the annealed primers are extended with the DNA polymerase.

The cycle of denaturation, annealing and DNA synthesis is repeated many times. The products of one round of amplification serve as templates for the next. Each successive cycle therefore essentially doubles the amount of the desired DNA product. The major product of this exponential reaction is a segment of the double stranded DNA whose termini are defined by the 5' termini of the oligo nucleotide primers and whose length is defined by the distance between the two primers (Sambrook *et al*. 1989). In case of reverse transcriptase PCR, the first strand synthesized by reverse transcriptase using mRNA as a template for the second strand synthesis. The two strands then undergo the routine exponential amplification reaction.

Primers for the PCR were designed on the basis of the sequence data of the coat protein gene of PRSV. The 5' upstream primer covered the start of CP coding region. It was designed as PRSVI and its sequence was: 5'd (**atcagtccaaaaatgaag**)3'. The 3' downstream primer covered the stop signal of the CP coding region. It was designated as PRSVII and its sequence was: 5'd (**atgcgcaactaaata**)3'

6.6.5.1 RT PCR Reaction

1XTAE

40mM Tris -acetate

0.1mM EDTA, pH-8.0

2X Reaction mix

RNA 8.5 µl

PRSVI 10.0 µM

PRSVII 10.0 µM

RT/TAQ Mix 2.0 µl (10 units/µg)

PCR reaction was set with the following conditions

i) denaturation at 94^0Cfor 5 minutes,

ii) annealing at 35^0C for 2 minutes

iii) extension at 72^0C for 2 minutes

A final extension step at 72^0C for 10 minutes was added. PCR was carried out for 25 cycles. The reaction products were analysed by electrophoresis on 1% agarose gel in 1X TAE buffer (40mM Tris -acetate and 0.1mM EDTA, pH-8.0)

The desired band was sliced out of the gel and taken in a pre-weighed eppendorf tube. Equal volume of phenol was added to the sample and vortexed for 15 secs. Aqueous phase was taken and equal volume of chloroform : isoamyl alcohol (24:1) was added and spun for 10000 rpm for 10 minutes at room temperature. The DNA was precipitated by adding 1/10 volume of NaOAC, 2.5 times volume of absolute alcohol and incubated at -20 ^{0}C overnight. Next day the sample was spun at 10000 rpm for 10 minutes. The precipitate was washed with 70% alcohol. The precipitate was then dried at room temperature and then dissolved in minimum amount of deionized water. Later it was observed on 1% agarose gel in 1XTAE .

6.6.6 Cloning of the RT-PCR amplified product (gene) in plasmid vectors

6.6.6.1 Bacterial transformation

JM109 is used to clone pGEM-3Zf (-) vector. This strain grows well and can be transformed well by avariety of methods. Also JM109 can be used for blue/white colour screening of the pGEM-3Zf plasmids (Hanahan 1985).

6.6.6.1.1 Preparation of competent cells

Single colony of *E.coli* JM 109 was inoculated in 2 ml LB medium and grown overnight at 37 ^{0}C. About 1% inoculum was added to 50 ml of fresh LB medium and grown for 2-3 h. Fresh cells were harvested by spinning at 4000 rpm for 10 minutes at 4^{0}C. The pellet thus obtained was washed with 20 ml ice cold solution of 100 mM $CaCl_2$ and centrifuged as above. The pellet was resuspended in 1ml of 100mM $CaCl_2$. This was then dispensed in 200 µl aliquots to eppendorf tubes and kept at 4 ^{0}C overnight.

Composition of LB medium *(*Sambrook *et al*. 1989)

Bacto-trypton-10g/l

Bacto yeast extract-5g/l

Nacl-10g/l

pH 7.0

6.6.6.1.2 Transformation of pGEM DNA into JM109 cells

Competent JM109 cells were thawn and mixed with 1µl (50 ng) pGEM 3Zf (-)DNA. The tubes were left on ice for 30 min. A heat shock at 42^{0}Cfor exactly 40sec was given after which the tubes were placed on ice for 2 mins. 800 µl of LB medium was added and the cells were allowed to recover at 37 ^{0}C for 1 hour. The cells were then plated on LB-agar amp^{50} IPTG-X-Gal plates and incubated at 37 ^{0}C overnight.

6.6.6.1.3 Screening for recombinants

Positive colonies were picked after screening by blue/white selection (disruption of lacZ gene). Each isolated colony was grown in 10ml LB-agar amp^{50} overnight. Plasmid DNA was isolated by the alkaline lysis method (Smbrook *et al*. 1989). Cells were pelleted down by centrifugation and re suspended in TEG buffer (50Mm Glucose, 25Mm Tris-HCl, pH 8.0). This was chilled on ice, 2 volumes of 0.2 M NaOH, 1%SDS was added and mixed well to allow the lysis of the cells. 1.5 volumes of 3 M potassium acetate, pH 4.8 was added and mixed well. The mixture was centrifuged at 16000Xg for 15 minutes at 4 ^{0}C and the supernetant was carefully collected. Plasmid DNA was precipitated out slowly with an equal volume of isopropanal at 4 ^{0}C for 4-6 hours. The precipitate was washed extensively with 70% ethanol, dried and dissolved. The DNA was digested with restriction enzymes *Kpn*I and *Bam* HI according to manufactures recommendation.

Reaction compoment

Plasmid DNA

Multicote ^{TM} buffer (25mM Tris acetate, (pH 7.5 at 37 ^{0}C) 100mM potassium acetate, 10mM magnesium acetate, 1mM DTT)

Bam HI (10units/µl)

*Kpn*I (10units/µl)

BSA (1 mg/ml)

Sterile deionized water

The RT-PCR product was also restriction digested with *Kpn*I and *Bam* HI as above and then ligated in to he pGEM 3Zf (-) vector. *The ligation reaction components were*

RT-PCR product

50ng pGEM 3Zf (-) DNA X *Kpn*I X *Bam* HI

Ligase (10units/µl)

Buffer

DNase free water

The ligation reaction was carried out at 16 ^{0}C overnight.

6.6.7 Transformation of the ligated plasmid into JM109 cells

The ligated product is then transformed in fresh competent E..coli JM109 cells in a similar process as described above (Sambrook *et al.* 1989). The cells were plated on LB-agar amp^{50} plates with IPTG and X-gal overnight. Positive colonies were screened by blue/white selection. Plasmid DNA was prepared (Sambrook *et al.* 1989) and digested with *EcoR* I and *Hind* III to release the insert.

Reaction component

DNA

M Buffer

RNAase

BSA

Water

*Eco R*I (10 units/µl)

Hind III (12 units/µl)

The vector pGEM3Zf (-) was also digested with the same, *EcoR* I and *Hind* III enzymes. The reaction product was analyzed by electrophoresis on 1% agarose gel in 1XTBE (10mM TRIS-Borate, 1mM EDTA, pH 8.0).

6.6.8 DNA sequencing and sequence analysis

DNA sequencing reactions were performed bidirectionally using ABI prism BigDye Terminator Cycle Sequencing kit from PE ABI. The primer extension products were separated by gel electrophoresis on an ABI sequencer. Sequence was assembled using Sequencher software. Nucleotide sequence of both strands was analyzed using FramePlot method for predicting protein coding region of bacterial DNA. Sequence comparisons and alignments were performed with the Basic Local Alignment Search Tool (BLAST; National center for Biotechnology information) and the Clustle X Multiple sequence alignment program (Baylor College of Medicine).

6.7 Results and Discussion

6.7.1 Total RNA observation

Denaturing formaldehyde-formamide gels run in MOPS/EDTA showed fluorescent several bands of RNA prepared from infected leaves of papaya. RNA marker size 0.24 to 9.49 kb were loaded alongside. The A260/A280 ratios was greater than 1.8 indicating only week contamination of polysaccharide and / or polyphenols and little or no protein contamination. The RNA integrity was judged by sharpness and presence of 28S and 18S ribosomal RNA band visualized on the denaturing RNA gel (Fig. 6.10A). A smear was observed on the denaturing gel that could be consequence of shear.

6.7.2 Isolation of PolyA$^+$ RNA from total RNA by Oligo (deoxythymidine) cellulose

Total RNA was first isolated from the tissues or cells. mRNA was then purified by PolyA$^+$ selection using oligo (dT) cellulose. This is necessary for all tissue sources rich in RNase (and some cell lines).

6.7.3 RT PCR

Some ribosomal RNA may remain mixed with mRNA. It's almost impossible to completely rid sample of all ribosomal RNA. The residual amount of rRNA left in sample does not interfere with reverse transcription.

PCR methodology has been adopted to generate cDNA fragments for specific sequences and to amplify them (Kawasaki 1990). In this method the first strand

synthesized by reverse transcriptase using mRNA as the template serves as the template for the second strand synthesis. The two strands then undergo routine exponential amplification reaction. Primers for RT PCR were designed on the basis of available sequence data of the coat protein gene of PRSV (Quemada *et al*. 1990, Slightom 1991). The 5' upstream primer was designated as PRSVI and its sequence was -5'd (**atcagtccaaaaatgaag)**3'.The 3' down stream primer was designated as PRSVII and its sequence was -5'd (**atgcgcaactaaata**)3'. The RT PCR product when visualized on gel shows a band of 800 bp which is nearer to the reported potyviral coat protein gene size (Fig.6.10 B). No nonspecific amplification was observed in this case.

6.7.4 Cloning of the amplified viral product

pGEM 3Z f (-) vector, a standard vector used for cloning purpose. It was linearized with enzymes *Kpn* I and *Bam*H I. The RT-PCR product was also restriction digested with *Kpn* I and *Bam*H I . The DNA was then purified and ligated to the vector. Cloning in pGEM 3Z f (-) vector enabled the use of T7 and SP6 sequencing primers both of which were readily available. The ligated plasmid was transformed into fresh competant *E. coli* JM109 cells. The clones were selected by blue white selection on ampicillin. Plasmid DNA from these clones was isolated and digested with EcoR I and *Hind* III (Fig. 6.10 C). The released insert from the correct clones (designated as pJB101) was about 800 bp as that of the RT-PCR product.

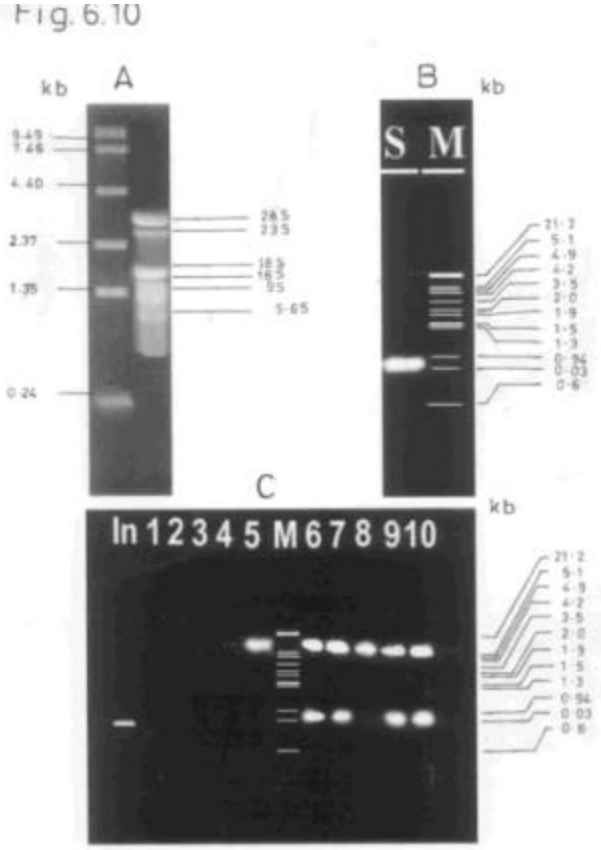


Fig. 6.10

A. Denaturing formaldehyde agarose gel electrophoresis of RNA in 1X MOPS/EDTA. Left to rightLane 1- Molecular weight marker , RNA Ladder (GIBCO BRL, USA). Sizes from top to bottom are 9.49,7.46,4.4,2.37,1.35 and 0.24 kb.Lane 2- Total RNA isolated from papaya PRSV infected leaves.

B. RT-PCR amplified product re-amplified by PCR. Lane 1(S)- re amplified RT-PCR product. Lane 2 (M)- λ DNA/*Eco*R I +*Hind* III marker. Sizes from top to bottom are 21.2,5.1,4.9,4.2,3.5,2.0,1.9,1.5,1.3,0.94,0.83 and 0.6 kb.

C. Cloning of the potyviral amplified product in pGEM3Zf (-) vector. In-Insert, M- λ DNA/*Eco*R I +*Hind* III marker. Sizes from top to bottom are 21.2,5.1,4.9,4.2,3.5,2.0,1.9,1.5,1.3,0.94,0.83 and 0.6 kb. Lane-1to 10- pJB101 1-10 digested with *Eco*R I and *Hind* IIIλ DNA/ E*co*RI+*Hind* III.

6.7.5 Sequencing

The approximate 800 bp putative PRSV CP insert was sequenced using AB1 BigDye Terminator system. The insert was determined to be 872 bp in size (Fig. 6.11), with a single ORF coding for 251 amino acids with an approximate relative molecular mass of 33.8 KD. The nuclear sequence was 40.7% G+C and 59.3% A+T.

6.7.6 Sequence analysis

Sequence analysis of the pJB101, 872 bp sequenced by BLAST 2 analysis revealed similarity with the CP gene of PRSV-P isolate i) South China coat protein (CP) gene, (Genbank Acc. No AF469066) - 89%, ii) Papaya ringspot virus isolate YS coat protein (CP) gene (Genbank Acc. No. AF469065) - 89%, iii) KPS coat protein mRNA, partial coding sequence (Genbank Acc. No. AF374862) - 91%, iv) Indonesia 2 coat

Fig. 6.11

Forward primer PRSVI

atcagtccaaaaatgaagctgtggatactggtttaaatgaaaaattcaaagaaaaggaaa

aacagaaagaaaaagaaaaagaaaaacaaaaagagaaagaaaaagacgatgctagtga

cggaaatgatgtgtcaactagcacaaaaactggagagagagatagagatgtcaatgttgg

gaccagtggaactttcactgttccgagaattaaatcatttactgataagatgattctaccgaga

attaagggaaagtctgtccttaatttaaatcacctacttcagtataatccgcaacaaattgacat

ttctaacactcgtgccactcagtcacaatttgagaagtggtatgagggagtgaggaatgatta

tggccttaatgataatgaaatgcaagtgatgctaaatggtttgatggtttggtgtatcgagaat

ggtacatctccagacatatctggtgtctgggttatgatggatggggaaacccaagttgattat

ccaatcaagcctttaattgagcatgctactccgtcatttaggcaaattatggctcactttagtaa

cgcggcagaagcatacattgcgaaaagaaatgctactgagaggtacatgccgcggtatgg

aatcaagagaaatttgactgacattagcctcgctagatacgctttcgacttctatgaggtgaat

tcgaaaacacctgatagggctcgcgaagcccacatgcagatgaaggctgcagcactgcg

aaacactagtcgcagaatgtttggtatggacggcagtgttagtaacaaggaagaaaacacg

gagagacacacagtggaagacgtcaatagagacatgcactctctcctgggg **atgcgcaa**

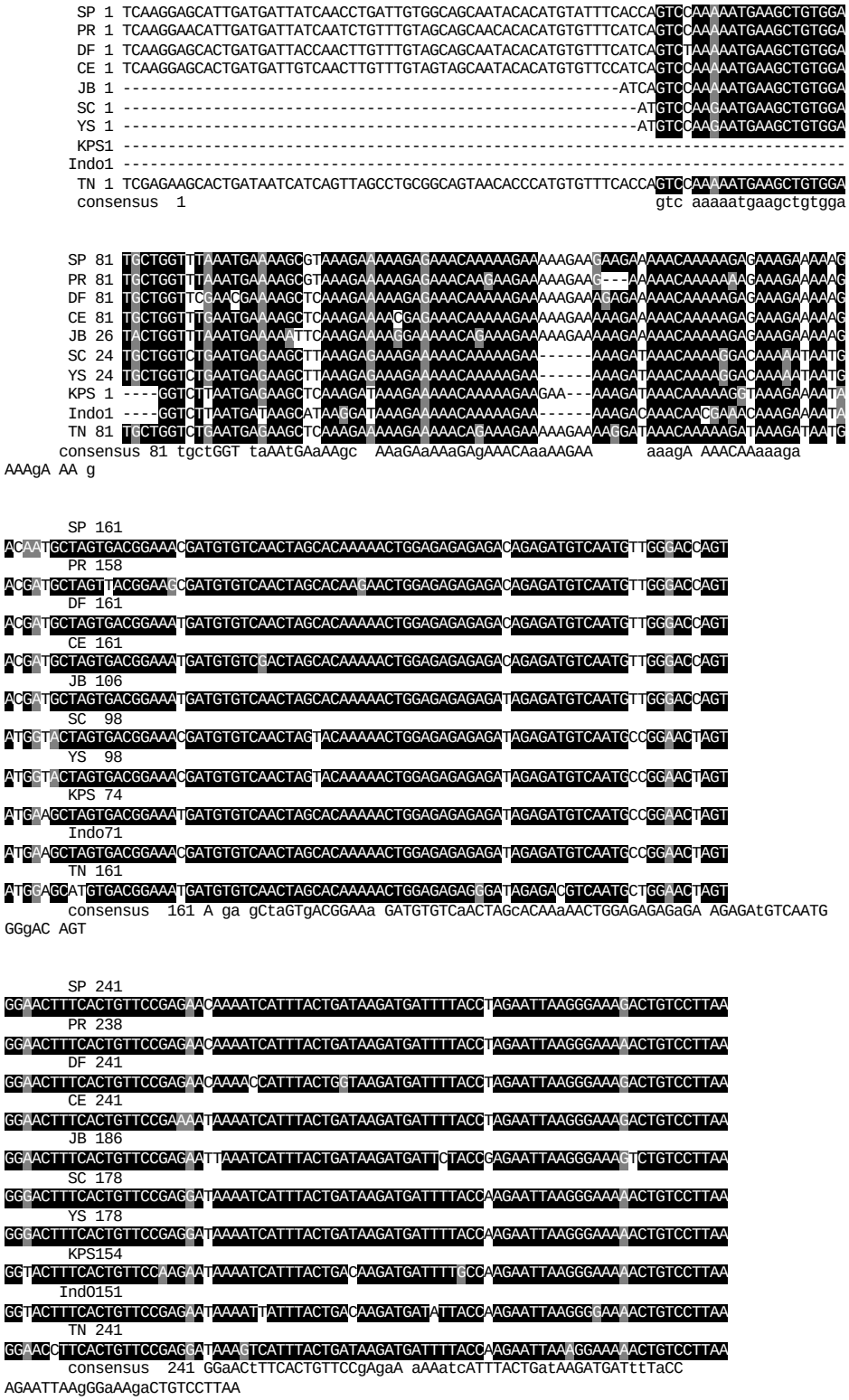
ctaaata

Reverse primer PRSVII

Fig. 6.11
Nucleotide sequence of the isolated 872 bp putative CP gene

protein mRNA, partial coding sequence (Genbank Acc. No. AF374865) - 90%, v)

Fig.6.12




SP 321 TTTAAATCATCTCCTTCAGTATAATCCGCAACAAATTGACATTTTTAACACTCGTGCCACTCAATCACAATTTGAGAAGT
PR 318 TTTAAATCATCTCCTTCAGTATAATCCGCAACAAATTGACATTTCTAATTACTCGTGCTACTCAATCACAATTTGAGAAGT
DF 321 TTTAAATCATCTCCTTCAGTATAACCCACAACAAATTGACATTTCTAACACTCGTGCCACTCAATCACAATTTGAGAAGT
CE 321 TTTAAATCATCTCCTTCAGTATAATCCACAACAAATTGACATTTCTAACACTCGTGCCACTCAATCACAATTTGAGAAGT
JB 266 TTTAAATCACCTACTTCAGTATAATCCGCAACAAATTGACATTTCTAACACTCGTGCCACTCAGTCACAATTTGAGAAGT
SC 258 TTTAAACAGTCTTCTTCAGTATAATCCGAAACAAATTGACATCTCTAACACTCGCGCCACTCAATCTCAATTTGAGAAGT
YS 258 TTTAAACAGTCTTCTTCAGTATAATCCGAAACAAATTGACATCTCTAACACTCGCGCCACTCAATCTCAATTTGAGAAGT
KPS234 TTTAAATCATCTTCTTCAGTATAATCCGCAACAAATAGACATCTCAAACACTCGTGCCACTCAATCTCAATTCGAAAAGT
Indo231 TTTAAATCATCTTCTTCAGTATAACCCGCAACAAATAGACATCTCAAACACTCGTGCCACTCAATCACAATTCGAAAAGT
TN 321 TTTGAATCATCTTCTTCAGTATAATCCGCAACAAATTGACATCTCAAACACTCGTGCCACTCAATCTCAATTTGAGAAGT
consensus 321 TTTaAAtcatCT CTTCAGTATAAtCCgcAACAAATtGACAT Tc AAcACTCGtGCcACTCAaTC
CAATTtGAgAAGT

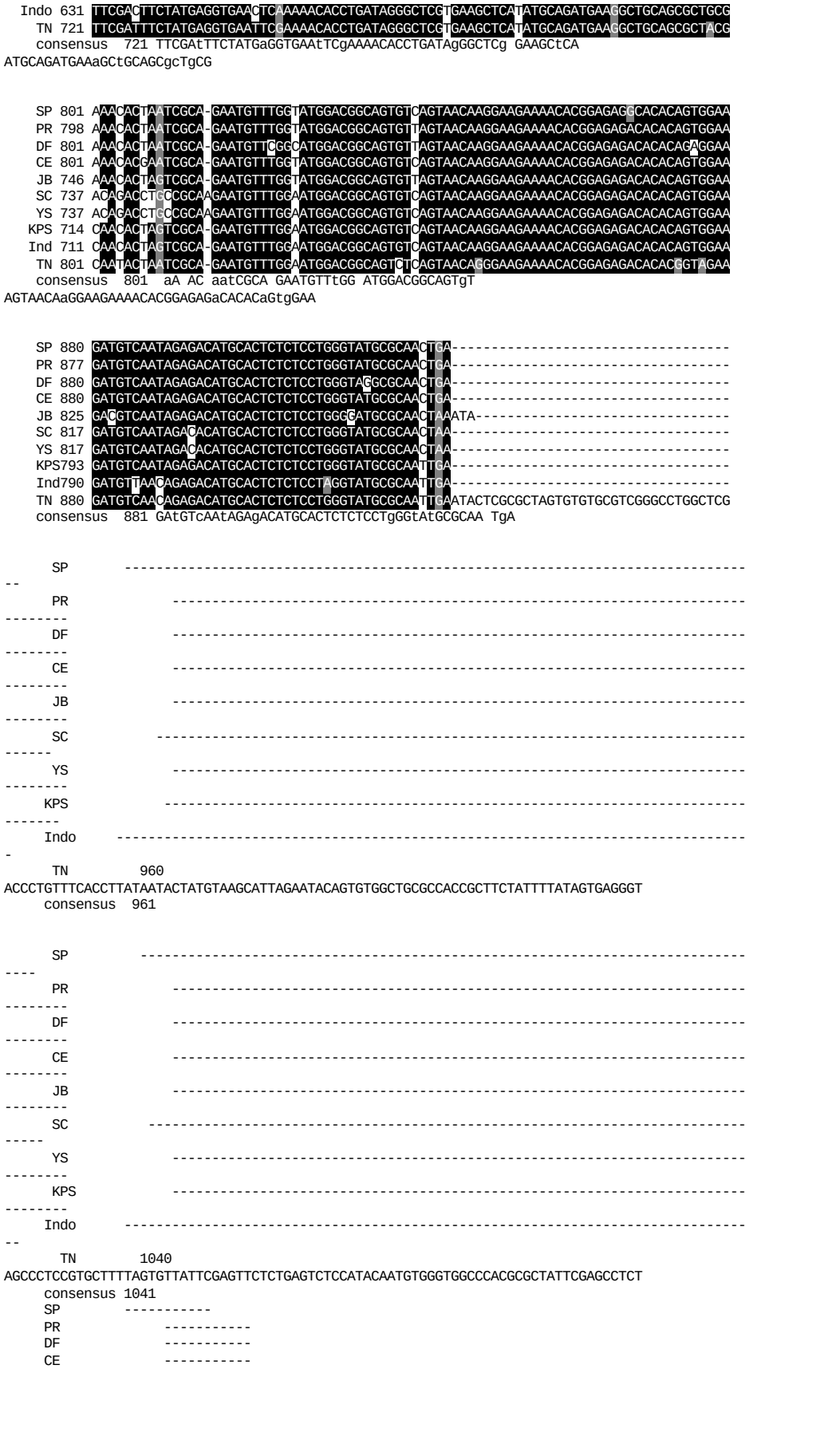

SP 401 GGTATGAGGGAGTGAGGAATGATTATGGCCTTAATGATAATGAAATGCAAATAATGCTAAATGGCTTGATGGTTTGGTGT
PR 398 GGTACGAGGGAGTGAGGAATGATTATGGCCTTAATGATGATGAGATGCAAATACGCGCTAAATGGTTTGATGGTTTGGTGT
DF 401 GGTATGAGGGAGTGAGGAATGATTATGGCCTTAATGACAATGAAATGCAAATAATGCTAAATGGCTTGATGGTTTGGTGT
CE 401 GGTATGAGGGAGTGAGGAATGATTATGGCCTTAATGATAATGAAATGCAAATAATGCTAAACGGCTTGATGGTTTGGTGT
JB 346 GGTATGAGGGAGTGAGGAATGATTATGGCCTTAATGATAATGAAATGCAAGTGATGCTAAATGGTTTGATGGTTTGGTGT
SC 338 GGTACGAGGGAGTGAGGAATGATTACGGCCTCAGTGATAACGAAATGCAAGTAATGTTAAACGGTTTGATGGTTTGGTGC
YS 338 GGTACGAGGGAGTGAGGAATGATTACGGCCTCAGTGATAACGAAATGCAAGTAATGTTAAACGGTTTGATGGTTTGGTGC
KPS314 GGTATGAGGGAGTGAGGAATGATTACGGTCTTAATGATAACGAAATGCAAGTGATGTTAAATGGTTTGATGGTTTGGTGC
Indo311 GGTATGAGGGAGTGAGGAATGATTATGGCCTTAATGATAACGAAATGCAAGTGATGTTAAATGGCCTTATGGTTTGGTGC
TN 401 GGTATGAGGGAGTGAGAAATGATTATGGCCTTAATGATAATGAAATGCAAGTGATGTTAAATGGTTTGATGGTTTGGTGT
consensus 401 GGTA GAGGGAGTGAGgAATGATTA GGcCTtAaTGAtaA GAaATGCAAaTaatG TAAA GG
tTgATGGTTTGGTG


SP 481 ATCGAAAACGGTACATCCCCAGACATATCTGGTGTCTGGGTTATGATGGATGGGGAAACCCAGGTTGATTATCCAATCAA
PR 478 ATCGAAAACGGTACATCTCCAGACATATCTGGTGTCTGGGTTATGATGGATGGGGAAACCCAGGTTGATTATCCAATCAA
DF 481 ATCGAGAACGGTACATCCCCAGACATATCTGGTGTCTGGGTTATGATGGATGGGGAAGCCCAAGTTGATTATCCAATCAA
CE 481 ATCGAGAATGGTACATCCCCAGACATATCTGGTGTCTGGGTTATGATGGATGGGGAAATCCAAGTTGATTATCCAATCAA
JB 426 ATCGAGAATGGTACATCTCCAGACATATCTGGTGTCTGGGTTATGATGGATGGGGAAACCCAAGTTGATTATCCAATCAA
SC 418 ATTGAAAATGGAACATCTCCAGACATATCTGGTGTCTGGGTAATGATGGATGGAGAAACTCAAGTCGATTACCCCATCAA
YS 418 ATTGAAAATGGAACATCTCCAGACATATCTGGTGTCTGGGTAATGATGGATGGAGAAACTCAAGTCGATTACCCCATCAA
KPS 394 ATCGAAAATGGAACATCCCCAGACATGTCTGGTGTCTGGGTGATGATGGATGGGGAAACCCAAGTTGATTATCCCATCAA
Indo 391 ATCGAAAATGGAACATCCCCAGACATATCTGGTGTCTGGGTGATGATGGATGGGGAAACTCAAGTCGATTATCCCATCAA
TN 481 ATCGAAAATGGTACATCTCCAGACATATCTGGTGTCTGGGTGATGATGGATGGGGAGACCCAAGTTGATTATCCCATTAA
consensus 481 ATcGAaAA GG ACATC CCAGACATaTCTGGTGTCTGGGT ATGATGGATGGgGAaac CAaGT
GATTAtCC ATcAA


SP 561 GCCTTTAATTGAGCATGCTACTCCGTCGTTTAGGCAAATTATGGCTCATTTCAGTAACGCGGCAGAAGCGTACATTGCAA
PR 558 GCCTTTAATTGAGCATGCTACTCCGTCGTTTAGGCAAATTATGGCTCATTTCAGTAACGCGGCAGAAGCATACATTACAA
DF 561 GCCTTTAATTGAGCATGCCACTCCGTCGTTTAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAAGCATACATTGCAA
CE 561 GCCTTTAATTGAGCATGCAACCCGTCGTTTAGGCAAATTATGGCTCACTTCAGTAACGCGGCAGAAGCATACATTGCAA
JB 506 GCCTTTAATTGAGCATGCTACTCCGTCATTTAGGCAAATTATGGCTCACTTTAGTAACGCGGCAGAAGCATACATTGCGA
SC 498 ACCTTTGATTGAGCACGCAACTCCTTCGTTTAGGCAGATTATGGCTCACTTCAGTAACGCGGCAGAAGCATACATCGCGA
YS 498 ACCTTTGATTGAGCACGCAACTCCTTCGTTTAGGCAGATTATGGCTCACTTCAGTAACGCGGCAGAAGCATACATCGCGA
KPS 474 GCCTTTGATGGAACATGCAACTCCTTCGTTGAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAGGCATACATCGCAA
Indo 471 GCCTTTGATGGAACATGCAACTCCTTCGTTGAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAGGCATACATCGCAA
TN 561 ACCTTTGATGGAACACGCAACTCCTTCATTTAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAGGCATACATCGCAA
consensus 561 gCCTTTaAT GAgCA GC ACtCC TCgTTtAGGCAaAT
ATGGCTCAcTTcAGTAACGCGGCAGAaGCaTACAT gCaA


SP 641 AGAGAAATGCTACTGAAAGGTACATGCCGCGGTATGGGATCAAGAGAAATTTGACTGACATTAGTCTCGCTAGATATGCT
PR 638 AGAGAAATGCTACTGAGAGGTACATGCCGCGGTATGGGATTAAAGAGAAATTTGACTGACATTAGTCTTGCTAGATATGCT
DF 641 AGAGAAATGCCACCGAGAGGTACATGCCGCGGTATGGAATCAAGAGAAATTTGACTGACATTAGCCTCGCTAGATATGCT
CE 641 AGAGAAATGCCACTGAGAGGTACATGCCGCGATATGGAATAAAGAGAAATTTGACTGACATTAGCCTCGCTAGATATGCT
JB 586 AAAGAAATGCTACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGAAATTTGACTGACATTAGCCTCGCTAGATACGCT
SC 578 AGAGGAATGCAACCGAGAGGTAGTTGCCGCGGTATGGAATCAAGAGAAATTTGACTGACATTAGTCTCGCTAGATATGCT
YS 578 AGAGGAATGCAACCGAGAGGTACTTGCCGCGGTATGGAATCAAGAGAAATTTGACTGACATTAGTCTCGCTAGATATGCT
KPS 554 AGAGGAATGCTACTGAAAGGTACATGCCGCGGTATGGAATCAAGAGGAATTTGACTGACATTAGTCTTGCTAGATATGCT
Indo 551 AGAGGAATGCAACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGGAATTTGACTGACATTAGTCTCGCTAGATATGCT
TN 641 AGAGGAATGCAACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGAAATTTGACTGACATTAGTCTCGCTAGATATGCT
consensus 641 AgAGaAATGC AC GAgAGGTACaTGCCGCGgTATGGaATcAAGAGaAATTTGACTGACATTAG
CTcGCTAGATAtGCT


SP 721 TTCGATTTCTATGAGGTGAATTCGAAAACACCTGATAGGGCTCGCGAAGCTCACATGCAGATGAAAGCTGCAGCGCTGCG
PR 718 TTCGATTTCTATGAGGTGAATTCGAAAACACCTGATAGGGCTCGCGAAGCTCACATGCAGATGAAAGCTGCAGCGCTGCG
DF 721 TTCGATTTCTATGAGGTGAATTCGAAAACACCTGATAGGGCTCGCGAAGCTCATATGCAGATGAAAGCTGCAGCGCTGCG
CE 721 TTCGATTTCTATGAGGTGAATTCGAAAACACCTGATAGGGCTCGTGAAGCTCATATGCAGATGAAAGCTGCAGCGCTGCG
JB 666 TTCGACTTCTATGAGGTGAATTCGAAAACACCTGATAGGGCTCGCGAAGCCCACATGCAGATGAAGGCTGCAGCACTGCG
SC 658 TTCGATTTCTATGCGGTGAATTCAAAAACACCTGATATGGCTCTCGAAGCTCACATGCAGATGAAAGCCGCAGCG-TGCG
YS 658 TTCGATTTCTATGCGGTGAATTCAAAAACACCTGATATGGCTCTCGAAGCTCACATGCAGATGAAAGCCGCAGCG-TGCG
KPS 634 TTCGATTTCTATGAGGTGAATTCAAAAACACCTGATAGGGCTCGTGAAGCTCATATGCAGATGAAGGCTGCAGCGCTGCG


Indo 631 TTCGAGTTCTATGAGGTGAACTCAAAAACACCTGATAGGGCTCGTGAAGCTCATATGCAGATGAAGGCTGCAGCGCTGCG
TN 721 TTCGATTTCTATGAGGTGAATTCGAAAACACCTGATAGGGCTCGTGAAGCTCATATGCAGATGAAGGCTGCAGCGCTACG
consensus 721 TTCGAtTTCTATGaGGTGAAtTCgAAAACACCTGATAgGGCTCg GAAGCtCA
ATGCAGATGAAaGCtGCAGCgcTgCG
SP 801 AAACACTAATCGCA-GAATGTTTGGTATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGGCACACAGTGGAA
PR 798 AAACACTAATCGCA-GAATGTTTGGTATGGACGGCAGTGTTAGTAACAAGGAAGAAAACACGGAGAGACACACAGTGGAA
DF 801 AAACACTAATCGCA-GAATGTTCGGCATGGACGGCAGTGTTAGTAACAAGGAAGAAAACACGGAGAGACACACAGAGGAA
CE 801 AAACACGAATCGCA-GAATGTTTGGTATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGTGGAA
JB 746 AAACACTAGTCGCA-GAATGTTTGGTATGGACGGCAGTGTTAGTAACAAGGAAGAAAACACGGAGAGACACACAGTGGAA
SC 737 ACAGACCTGCCGCAAGAATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGTGGAA
YS 737 ACAGACCTGCCGCAAGAATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGTGGAA
KPS 714 CAACACTAGTCGCA-GAATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGTGGAA
Ind 711 CAACACTAGTCGCA-GAATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGTGGAA
TN 801 CAATACTAATCGCA-GAATGTTTGGAATGGACGGCAGTCTCAGTAACAGGGAAGAAAACACGGAGAGACACACGGTAGAA
consensus 801 aA AC aatCGCA GAATGTTtGG ATGGACGGCAGTgT
AGTAACAaGGAAGAAAACACGGAGAGaCACACaGtgGAA
SP 880 GATGTCAATAGAGACATGCACTCTCTCCTGGGTATGCGCAACTGA----------
PR 877 GATGTCAATAGAGACATGCACTCTCTCCTGGGTATGCGCAACTGA----------
DF 880 GATGTCAATAGAGACATGCACTCTCTCCTGGGTAGGCGCAACTGA----------
CE 880 GATGTCAATAGAGACATGCACTCTCTCCTGGGTATGCGCAACTGA----------
JB 825 GAGGTCAATAGAGACATGCACTCTCTCCTGGGGATGCGCAACTAAATA----------
SC 817 GATGTCAATAGACACATGCACTCTCTCCTGGGTATGCGCAACTAA----------
YS 817 GATGTCAATAGACACATGCACTCTCTCCTGGGTATGCGCAACTAA----------
KPS793 GATGTCAATAGAGACATGCACTCTCTCCTGGGTATGCGCAATTGA----------
Ind790 GATGTTAACAGAGACATGCACTCTCTCCTAGGTATGCGCAATTGA----------
TN 880 GATGTCAACAGAGACATGCACTCTCTCCTGGGTATGCGCAATTGAATACTCGCGCTAGTGTGTGCGTCGGGCCTGGCTCG
consensus 881 GAtGTcAAtAGAgACATGCACTCTCTCCTgGGtAtGCGCAA TgA
SP ----------
--
PR ----------

DF ----------

CE ----------

JB ----------

SC ----------

YS ----------

KPS ----------

Indo ----------
-
TN 960
ACCCTGTTTCACCTTATAATACTATGTAAGCATTAGAATACAGTGTGGCTGCGCCACCGCTTCTATTTTATAGTGAGGGT
consensus 961
SP ----------

PR ----------

DF ----------

CE ----------

JB ----------

SC ----------

YS ----------

KPS ----------

Indo ----------
--
TN 1040
AGCCCTCCGTGCTTTTAGTGTTATTCGAGTTCTCTGAGTCTCCATACAATGTGGGTGGCCCACGCGCTATTCGAGCCTCT
consensus 1041
SP ----------
PR ----------
DF ----------
CE ----------

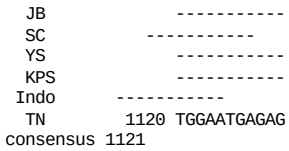


Fig. 6.12

Multiple sequence alignment of the 872 bp nucleotide sequence of PRSV CP insert with SC biotype, (Genbank Acc. No AF469066), YS biotype (Genbank Acc. No. AF469065), KPS biotype (Genbank Acc. No. AF374862), Indonesia biotype (Genbank Acc. No. AF374865), SP biotype (Genbank Acc. No. AF344642), PR biotype (Genbank Acc. No. AF344643), TN biotype (Genbank Acc. No. AY027812), DF biotype (Genbank Acc. No AF344649) and CE biotype (Genbank Acc. No. AF344648).

Fig. 6.13

5'3' Frame 3

Q S K N E A V D T G L N E K F K E K E K Q K E K E K E K Q K E K E K D
D A S D G N D V S T S T K T G E R D R D V N V G T S G T F T V P R I K S
F T D K M I L P R I K G K S V L N L N H L L Q Y N P Q Q I D I S N T R A T
Q S Q F E K W Y E G V R N D Y G L N D N E M Q V M L N G L M V W C I
E N G T S P D I S G V W V M M D G E T Q V D Y P I K P L I E H A T P S F R
Q I M A H F S N A A E A Y I A K R N A T E R Y M P R Y G I K R N L T D I
S L A R Y A F D F Y E V N S K T P D R A R E A H M Q M K A A A L R N T
S R R M F G M D G S V S N K E E N T E R H T V E D V N R D M H S L L G
M R N

Fig. 6.13
Deduced amino acid sequence of the isolated 872 bp putative CP gene. Arrow indicates the cleavage site between NIa and CP region of the PRSV insert.

Fig. 6.14

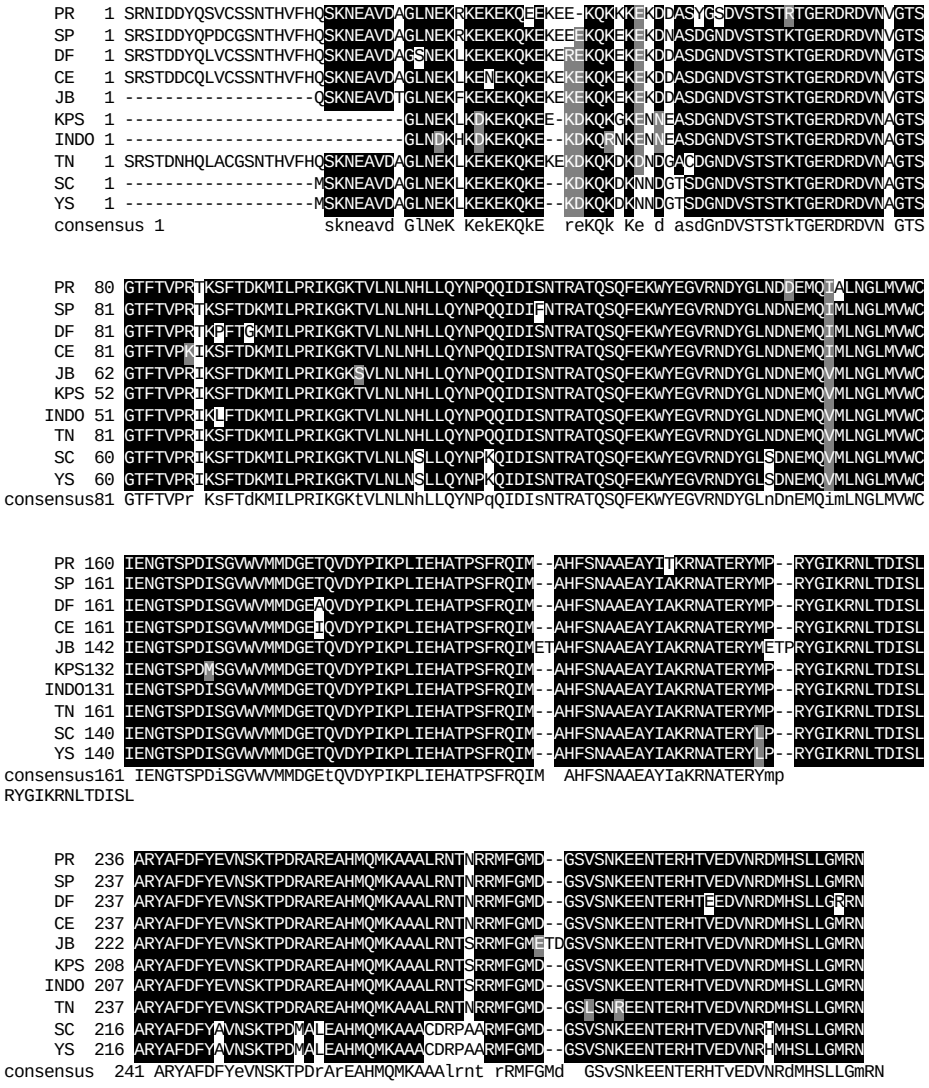


Fig. 6.14

Deduced multiple sequence alignment of the 251 amino acid (with relative molecular mass of 33.8 KD) sequence of PRSV CP insert with SC biotype, (Genbank Acc. No AF469066), YS biotype (Genbank Acc. No. AF469065), KPS biotype (Genbank Acc. No. AF374862), Indonesia biotype (Genbank Acc. No. AF374865), SP biotype (Genbank Acc. No. AF344642), PR biotype (Genbank Acc. No. AF344643), TN biotype (Genbank Acc. No. AY027812), DF biotype (Genbank Acc. No AF344649) and CE biotype (Genbank Acc. No. AF344648).

SP biotype P coat protein gene, partial coding sequence (Genbank Acc. No. AF344642) - 95%, vi) PR biotype P coat protein gene, partial coding sequence (Genbank Acc. No. AF344643) - 94%, vii) TN coat protein gene, partial coding sequence (Genbank Acc. No. AY027812) - 91%. The sequence similarity with the PRSV -W isolate CP gene was i) 94% with DF biotype W coat protein (CP) gene, partial coding sequence (Genbank Acc. No AF344649) and ii) 95% with CE biotype W coat protein (CP) gene, partial coding sequence (Genbank Acc. No. AF344648) (Fig. 6.12).

The deduced amino acid sequence (Fig. 6.13) similarity with PRSV-P isolate was 83% with South China coat protein (CP) gene, (Genbank Acc No. AF469066), ii) 83% with YS coat protein (CP) gene (Genbank Acc No. AF469065), iii) 95% with KPS coat protein mRNA, partial coding sequence (Genbank Acc No. AF374862), iv) 95% Indonesia 2 coat protein mRNA, partial coding sequence (Genbank Acc No. AF374865,) v) 88% SP biotype P coat protein gene, partial coding sequence (Genbank Acc No. AF344642), vi) 86% with PR biotype P coat protein gene, partial coding sequence (Genbank Acc No. AF344643), vii) 87% with TN coat protein gene, partial coding sequence (Genbank Acc No. AY027812) and with PRSV W isolate was i) 86% with DF biotype W coat protein (CP) gene, partial coding sequence (Genbank Acc No. AF344649 ii) 88% with CE biotype W coat protein (CP) gene, partial coding sequence (Genbank Acc No. AF344648) (Fig. 6.14).

As reported earlier the NIa -CP cleaving junction Q-S is present in the isolated clone (Quemada *et al.* 1990; Slightom 1991).

6.8 Conclusion

The PRSV CP gene isolated in our laboratory showed a nucleotide size of 872 bp with a relative molecular mass of 33.8 KD and a capacity to code for 251 amino acids. Multiple sequence analysis of pJB101 with BLAST and CLUSTLE showed a nucleotide sequence similarity of 94-95% with PRSV -W strain and 89-95% similarity with PRSV -P strain. The deduced amino acid sequence also showed a sequence similarity of 86-88% with PRSV-W type and 83-95% with PRSV-P type.

SUMMARY

Biotechnology is now the cutting edge of plant science offering new techniques, applications and opportunities for plant improvement. Application of biotechnological tools in crop improvement programs can be effective in three different complimentary ways: 1) by speeding up the process of conventional breeding, 2) development of novel genotypes through recombinant DNA technology and 3) by creating genetic variability through tissue culture. It can provide solutions to problems like disease and pest management, biotic and abiotic stresses, and can be useful in increasing the productivity of specific crop

Papaya one of the important commercial plantation fruit crops of the world is valued for its high content of vitamins and minerals in the fruit flesh, and the enzyme papain and other by-products. Papaya belongs to the genus *Carica* under the family *Caricaceae*. The crop is highly popular and grown in almost all the tropical and subtropical countries around the globe.

The total production of papaya in India is about 644,000 Mt from a cultivated area of 57,000 hectares (FAO 2000). The crop is gown all over the country and is available round the year. The state of Karnataka in India is the lead producer of papaya in India, followed by the states of Orissa and West Bengal.

Papaya is susceptible to a number of diseases. Among these, the aphid transmitted, Papaya Ring spot Virus (PRSV) causes severe damage to the crop. Recently, major efforts have been directed towards the introduction of new agricultural traits into papaya through genetic engineering to solve this problem. However, majority of the published reports on papaya pertain to exotic cultivars not grown in India. Since, India is one of the major papaya producers, concerted efforts are underway to develop transgenic papaya using local Indian cultivars as the starting material..

An efficient plant regeneration protocol is a major pre-requisite for development of transgenic plants. At the time of initiation of this study, no reports on *in vitro* somatic embryogenesis and multiple shoot formation from embryo axes explant of the most commonly grown local papaya cultivars existed. We have selected cultivars Honey Dew, Washington and Co-2 on the basis of their popularity and availability of seeds in the local market. Therefore, this study was initiated with an objective of developing a highly reproducible and efficient *in vitro* plant regeneration method from the cultivars that are

commonly grown in India. The study also aimed to standardize *Agrobacterium* mediated transformation of papaya explants and studies relating to isolation and cloning of the PRSV coat protein (CP) gene so that Indian cultivars of papaya resistant to PRSV could be developed.

The findings of the entire work are summarized below:

A: Comparative studies on *in vit*ro and *in vivo* seed germination of papaya

1. A protocol of *in vitro* seed germination of papaya was developed.
2. Germination of papaya seeds in *in vtiro* conditions resulted in better response than *in vivo* seed germination in terms of percent germination and also the number of days required for germination.
3. Presowing treatment with 200 ppm GA_3 for a period of 24 hrs was found to be ideal for increasing the germination percentage.
4. Optimum seed germination was at 30^0C.
5. TDZ as growth regulator (0.1-4.0 mg/l, best being 0.2mg/l) could also be suitably used to enhance the papaya seed germination rate in *in vitro* condition.
6. Among the various parameters tested for optimizing seed germination, 16 h photoperiod compared to darkness**,** agar as gelling agent and filter paper as support material could increase the germination percent in our study.
7. Cefotaxime and carbenicillin, most commonly used in tissue culture studies were found to exhibit a positive influence in papaya seed germination.
8. Among all the cultivars tested for papaya seed germination, cultivar Honey Dew was found to be the best responsive both in *in vitro* and *in vivo* condition.

B: Induction of somatic embryogenesis and plant regeneration in papaya.

1. A plant regeneration system *via* somatic embryogenesis was developed using immature embryo explant of cultivars Honey Dew, Washington and Co-2 and also from mature embryo explant of cultivars Honey Dew and Co-2.
2. The somatic embryos were induced from the shoot pole region of immature embryo axes when cultured on MS basal medium supplemented with a varied range of 2,4-D (2-25 mg/l) or 2,4,5-T (0.5-8.0 mg/l), or Picloram (0.5-10mg/l) or Dicamba (1-

15mg/l). MB liquid basal medium supplemented with 2,4-D or 2,4,5-T (1-5 mg/l) could also induce the somatic embryos from apical end of mature embryo axes explant in our study.

3. Auxin 2,4,5-T at 3.0 mg/l was found to be optimum for somatic embryo induction in case of both immature and mature embryos.
4. Although maximum response for somatic embryo induction was observed with 2,4-D (2.0mg/l) on all the three cultivar tested (Honey Dew - 86.36%, Co-2 - 76.22% and Washington-93.61%), maximum globular embryos per explant was observed with 3.0mg/l 2,45-T (Honey Dew - 29.33, Co-27.66 and Washington- 33.00).
5. Cotyledonary staged embryos could be obtained in the same somatic embryo induction medium in case of both the explants (immature and mature embryo) tested for somatic embryogenesis.
6. Maturation of the somatic embryos was achieved on MS basal medium supplemented with ABA (0.1mg/l) within 5-7 days of incubation.
7. In case of immature embryo, maximum germination (Honey Dew-70.66 %, Washington-72.0 % and Co-2 73.33 %) of the somatic embryos derived from 2,4-D medium, occurred in MS basal medium supplemented with GA_3 (1mg/l).
8. However, embryos obtained on medium containing 2,4,5-T (3.0 mg/l) germinated maximally in either phytohormone free MS basal medium (71.33 % in Honey Dew-, 59.33 % in Co-2) or in medium supplemented with GA_3 at 1mg/l (73.3 % in Washington).
9. Germination and conversion of the somatic embryos was achieved on MS basal medium supplemented with GA_3 (1mg/l) in case of embryos derived from mature embryo explant.
10. On an average 60 % of the somatic embryo derived seeding survived in cultivar Honey Dew, 40 % in Co-2 and 20 % in Washington in case of immature embryo explant whereas 25 % of the somatic embryo derived seedling survived in case of mature embryo explant derived embryos.
11. The histological and scanning electron microscopy studies confirmed the direct origin of the somatic embryos.

12. Effect of different basal media, type of explant, carbohydrate source, culture condition and gelling agent was studied on somatic embryo induction from immature embryo axes. In case of mature embryos, different parameters like basal medium, pH, $AgNO_3$, temperature and physical state of the medium (solid/liquid) were also evaluated to enhance the frequency of induction of somatic embryos.
13. In our study, MS basal medium, embryo axes explant, 3% sucrose, incubation of explants in dark and media with agar (0.75%) as gelling agent were found to be optimum for somatic embryo induction from immature embryo axes.
14. In case of mature embryo axes, explants cultured in MB liquid medium at pH of 5.8 and incubated at 25±2 ^{0}C in dark induce maximum somatic embryos from cultivar Co-2.

C: *In vitro* induction of multiple shoots and plant regeneration from embryo axis and shoot tip explant of papaya

1. A protocol of plant regeneration *via* organogenesis was developed for immature embryo axes of cultivars Honey Dew, Washington and Co-2. A system of shoot tip multiplication was developed for cultivars Honey Dew and Co-2.
2. Both immature embryo axes and cotyledon explant produced shoot buds on MBG basal medium supplemented with TDZ (0.1-5.0 mg/l). However, when BAP and NAA (0.05-2.0mg/l and 0.1-0.5mg/l respectively) were tested in combination, only the immature embryo explants respond by producing shoot buds.
3. Maximum number of shoots were produced upon culture of immature embryo axes explants of all the three cultivars on MBG basal medium containing TDZ (0.5 mg/l) and in presence of a combination of BAP and NAA at (1.0 mg/l and 0.1 mg/l respectively).
4. Maximum multiple shoot proliferation from the seedling shoot tip explants was also achieved in MBG basal medium containing BAP and NAA (0.5 mg/l and 0.1 mg/l).
5. Among the various parameters studied on shoot proliferation from the shoot tip explant, explants excised from 30 days old seedling and cultured in glass bottle containing MBG medium favoured the maximum induction of shoots.
6. *De novo* origin of the shoot buds from immature embryo explant was confirmed by histological and SEM study.

7. Elongation of shoot buds (obtained from embryo axis explants) was achieved on MBG medium supplemented with GA_3 (2.0 mg/l).
8. Maximum rooting of the plantlets (93.33 %) was achieved on MBG basal medium supplemented with IBA (3 mg/l).
9. *In vitro* regenerated plants, 76 % (derived from embryo axis) and 85% (derived from shoot tip) survived after hardening under greenhouse condition

D: Transformation studies in papaya

The present study was carried out with the objectives of optimization of co-cultivation with *Agrobacterium* to achieve transformation in papaya cultivars using embryo axes explants.

1. Immature embryos of cutlivar Honey Dew were used for *Agrobacterium* mediated transformation.
2. *Agrobacterium tumefaciens* LBA4404 containing the plasmid pGV2260-35S GUSINT was used as a vector for transformation.
3. LD_{50} for selection of transformed shoots was found to be 100 mg/l kanamycin.
4. Transformation frequency was influenced by the period and mode of treatment of *Agrobacterium* strain
5. GUS expression in callus tissue was achieved when explants are treated with *Agrobacterium* for a period of 5,10 and 20 minutes and incubated in dark for 72 h.
6. Explants treated with *Agrobacterium* and co-cultivated for 72 h in light (27 $\mu Em^{-2}s^{-1}$) did not favour transformation event.
7. Maximum number of explants showing expression of GUS gene was obtained when these were incubated with *Agrobacterium* for *5* minutes and co-cultivated for 72 hours in dark.
8. Occurrence of the transformation event was confirmed by histochemical means..
9. The PRSV CP gene was isolated and its sequencing revealed it to be 872 bp in size coding for a protein of 251 amino acids and a relative molecular mass of 33.8 KD..
10. Multiple sequence alignment analysis of pJB101 with BLAST and CLUSTLE showed a nucleotide sequence similarity of 94-95% with PRSV -W strain and 89-95% similarity with PRSV -P strain.

11. The deduced amino acid sequence also showed a sequence similarity of 86-88% with PRSV-W type and 83-95% with PRSV-P type.

The work presented in this thesis, forms the basic framework for biotechnology of papaya. The standardized regeneration protocols could be successfully used in genetic transformation of Indian cultivars of papaya. Also results obtained from *Agrobacterium* transformation study and our efforts towards isolation and cloning of CP gene could be utilized in development of transgenic papaya resistant to Papaya Ring Spot Virus (PRSV).

BIBLIOGRAPHY

Agrawal DC, Banerjee AK, Kedari P, Jacob S, Hazra S, Krishnamurthy KV (1998) J Plant Physiol 152:580-582

Ahuja MR (1986) Curr Sci 55:217-224

Ahuja MR (ed) (1993) Micro propagation of woody plants. Kluwert Academic Publishers Dordrecht

Aleman-Verdaguer M, Goudou-Urbino C, Dubern J, Beachy RN, Fauquet C (1997) J.Gen Virol 78:1253-1294

Allan P (1964) Farm S Afr 101:1-6

Allan P (1976) Acta Hortic.57: 97-103

Allison RF, Dougherty WG, Parks TD, Johnston RE, Kelly ME, Armstrong FB (1985) Viology 147: 309-316

Ammirato PV (1974) Bot Gaz 135:328-337

Ammirato PV (1983) Embryogenesis. In: Evans DA, Sharp WR, Ammirato PV, Yamada Y (eds) Handbook of plant cell culture, vol 1. MacMillan Publishing Co, New York, pp 82-123

Ammirato PV and Styer DJ (1985) Strategies for large scale manipulation of somatic embryogenesis in suspension culture. In: Zailtin M, Day P, A Hollaaender (eds) "Biotechnology in Plant Sci" Relevance to agriculture in the 1980's, Academic Press New York pp 161-178

Anonumous (2000) Dept of Agriculture, Maharashtra. http:// agri.mah.nic.in/agri/main page E. html, sept 2000.

Anonumous (2001) www.ansci.cornell.edu/plants/medicinal/papaya.html

APS (1998) (The American phytopathological society) net feature Sept 1-30

ARC (2001) ARC-ITSC, Zambia. www.ardc.agric.za/institutes./itsc/main/papaya

Arnold R.M., Slyker JA, Gupta TH, (1996) J Plant Physiol 148:677-683.

Arora IK, Singh RN (1978a) Sci Hortic 8(4):357-361

Arora IK, Singh RN (1978b) Curr Sci 47:867-868

Arriola MC, Calzada JF, Menchu JF, Rolz C, Garcia R (1980). Papaya In: Nagy S Shaw PE (eds) Tropical and Subtropical fruits, AVI West port pp 316-340

Atreya PL, Lopez-Moya JJ, Chu M, Atreya CD, Pirone TP (1995) J. Gen Virol 76:265-270

Atreya PL, Lopez-Moya JJ,Chu M., Atreya CD, Pirone TP (1991) Proc. Natl. Acad. Sci USA 88: 7887-7891

Baburaj S, Dhamotharan R, Santhaguru K (1987) Curr Sci 56:194-196

Badillo VM (1967) Esquema de las Carica ceae. Agron Trop 17:245-272.

Badillo VM (1971) Monografia dela Familia Caricaceeae. Asociacionde Profesores. Maracay, Venezuela.

Baker B, Zambryski P, Staskawicz B, Dinesh-Kumar SP (1997) Science 276: 726-733

Baker CM, Wetzstein HY (1992) Plant Cell Rep 11:71-75

Banerjee AK (2001). Ph.D. Thesis, Biotechnology Dept, University Of Pune, India.

Bastian L, Maene L, Harbaoui, Van Sumare C, Vande Castelle KL, Deberg PC (1983) Med Fac Landbouww Rijsuniv Gent 48:13-24

Bateson M, Dale J (1992) Arch Virol 123:101-109

Bateson M, Henderson J, Chaleeprom W, Gibbs AJ, Dale J (1994) J Gen Virol 75:3547-3553.
Beachy R, Loesch-Fries S, Tumer N (1990) Ann Rev Phytopatho 28:451-474
Bewley JD (1980). Physiol Plant 49:277-280
Bewley JD, Black M (1994) Seeds. Plenum Press New York and London
Bhagwat B, Vieira LGE, Erickkson LR (1996) Plant Cell Tissue Organ Cult 46:1-7
Bhaskaran S, Prabhudesai VR (1988) Tissue culture of plantation crops. In V. Dhawan (ed) Alications of biotechnolofy in forestry an dhorticulture, Plenum press, New York.
Bhattacharjee S, Khan HA, Reddy PV (1999). Indian Agriculture 43(1&2):79-83
Bhattacharya J, Khuspe SS (1999). In: International conference on life science in next millenium, Hyderabad, India, Dec11-14
Bhattacharya J, Khuspe SS (2001). Sci Horti 91:39-49
Bhattacharya J, Khuspe SS, Renukdas NN, Rawal SK (2002) Ind J Exp Biol 40: 624-627
Blumberg DD (1987) Methods Enzymology 152:20-24
Brar DS and Khush GS (1994) In Mechanism of improved plant growth and productivity (ed Basara AS) 229-276.
Brassard N, Brissette L, Lord D, Laliberte S (1996) Plant Cell Tissue Org Cult 44:37-44.
Bruijne E.de, ED Langhe, RU Rijck (1974) Rijksuniversiteit Gent 39(2):637-645
Bunn E, Dixon Kwand Langley MA (1989) Plant Cell Tissue Organ Cult 19:77-84
Burikam S, Chommalee V, Attathom S (1988). Kasetsart Journal. Natural Sciences 22(5): 1-6
Cabrera Ponce JL, Vegas Garcia A, Herrera Estrella L (1995) Plant Cell Rep15:1-7
Cabrera Ponce JL, Vegas Garcia A, Herrera Estrella L (1996) In Vito Cell Dev Biol Plant 32 P:86-90
Cai WQ, Gonsalves C, Tenent P, Fermin G, Jr.Souza M,Sarindu N, Jam FJ, Zhu HY, Gonsalves D (1999) In Vito Cell Dev Biol Plant 35:61-69
Carman JG (1989) In Vitro Cell Dev Biol 25:1155-1162
Carman JG (1990) In Vitro Cell Dev Biol 26:746-753
Casse Deelbart F (1989)J. Gen. Virology70: 935-937
Castillo B, Smith MAL, Yadava UL (1998) J Hort sci Biotechnol. 73: 307-311.
Castillo B, Smith MAL, Yadava UL (1998) Plant Cell Rep 17:172-176
Chacko EK, Singh RN (1966) Trop Agril 43(4): 341-346
Chalupa V (1988) Biol Plant 30:414-421
Chan L, Teo CKH, Chan LK (1994) Pertanika journal of Tropical Agricultural Science 17(2):103-106
Chandel SRS (1993) A handbook of agricultural statistics. Achal prakashan mandir, Kanpur, India, pp. B 35-36
Chang SS, Park SK, Kim BC, Kang BJ, Kim DU, Nam HS (1994) Plant J 5:551-558
Chang WC, Hsing YI (1980) Theor Appl Genet. 57:133-135
Chee PP (1994) Hort Sci 29(6):695-697
Chen FC, Kuo MH (1988) Ovule and Ovary Culture in papaya and somatic embryogenesis. In: Ma S-S, Shii G-T Chang WC Chang TL (eds) Proc Symp Tissue Culture of horticultural Crops National Taiwan University Taipei, March 8-9, 1988. Department of Horticulture, National Taiwan University, 1991, pp 50-61

Chen G,Ye CM, Huang JC , Yu M, Li BJ (2001) Plant Cell Rep 20:272-277
Chen MH (1988) Tissue culture and ringspot resistant breeding in papaya. In: MA S-S ,Shii CT,Chang WC, Chang TL (ed) Proc sym Tissue culture of horticultural crops, National Taiwan University, Taipei, March 8-9
Chen MH (1988b) Somatic embryogenesis and plant regeneration in Carica papaya L. tissue culture derived from root explant. In: Ma S-S, Shii C-T Chang WC Chang TL (eds) Proc Symp Tissue Culture of horticultural Crops National Taiwan University Taipei, March 8-9, 1988. Department of Horticulture, National Taiwan University, 1991, pp 230-233
Chen MH, Chen CC (1992) Plant Cell Rep 11:404-407
Chen MH, Chen CC, Wang DN, Chen FC (1991) Can. J Bot 69:1913-1918
Chen MH, Wang PJ, Maeda E (1987) Plant Cell Rep 6:348-351
Cheng YH, Yang JS, Yeh SD (1996) Plant Cell Rep 16:127-132
Chengalrayan (1997) Ph.D. Thesis, Biotechnology Dept, University Of Pune, India
Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Biochemistry 18:5294-5299
Christou P (1996) Trends Plant Sci 1:423-431
Citovsky V, Warnick D, Zambryski P (1994) Proc Natl Acad Sci USA 91:3210-3214
Conover, RA (1964) FIa State Hort Soc 77:440-444
Constabel F, Shyluk JP (1994) Initiation, nutrition and maintenance of plant Cell and Tissue Culture In: Plant Cell and Tissue Culture. Vasil IK and TA Thorpe (eds) Kluwer Academic Publishers, Dordrecht. pp 3-15
Cousins YL, Lyon BR, Llewellyn DJ (1991) Aust J Plant Physiol 18:481-494
Das S, Timir BJ, Sumita J (1996) Plant Cell Rep 15:615-619
Davies DR, Hamilton J, Mullineaux P (1993) Plant Cell Rep 12:180-183
De Block M, Herrera Estrella L, Van Montagu MC, Caplan AB (1990) Plant Cell 2:591-602
De Bruijne E, DeLanghe E, Van Rijck R (1974) Actions of hormones and embryoid formation in callus cultures of Carica papaya. In internationa l smposium on crop protection, Fytofarmacie en Fytiatric Rijkslandsbouwhoogeschool Medellelingen 39:637-645.
Debergh PC (1983) Med Fac Land bouww Rijsuniv Genet 48: 13-24
Dekeyser RA, Claes B, De Rycke RMU, Habets ME, Van Montagu MC, Caplan AB (1990) Plant Cell 2:591-602
Depauw MA, Remphrey WR (1993) Can J Bot 71: 879-885
Deproft MP, Maene LJ, Debergh PC (1985) Physiol Plant 65:375-379
Doem CM, Lapidot M, Beachy RN (1992) Cell 69: 221-224
Dolja VV, Haldemen CahillR, Montgomery AE, Vandenbosch KA, Carrington JC (1995) Virology 206: 1007-1016
Dolja VV, Haldemen CahillR, Robertson NL, Dougherty WG, Carrington JC (1994) EMBRO J 13: 1482-1491
Dongall DK, Verma DC (1978) In vitro Cell Dev Biol 24:180-182
Doty SL, Yu MC, Lundin JI, Heath JD, Nester EW (1996) J Bacteriol 178:961-970
Dougherty WG, Allison RF, Parks TD, Johnston RE, Field MJ, Armstrong FB (1985) Virology 146: 282-291

Draper J, Davey MR, Freeman JP, Cocking EC, Cox BJ (1982) Plant and Cell Physiol 23:451-458
Drew RA (1987) J. Hortic Sci 62:551-556
Drew RA (1988) Hort Sci 23:609-611
Drew RA, Considine JA, McComb JA (1993) Aust J Bot 41:739-748
Drew RA, Miller RM (1990) Plant Cell Tissue Org Cult 21:39-44
Drew RA, Smith NG (1986) J Hortic Sci 61:535-543
Duke JA (1984) Borderline herbs CRC Press. Boca Raton, FL.
Durham RE, Parrott WA (1992) Plant Cell Rep 11:122-125
Dye F, Berthelot K, Griffon B, Delay D, Delmotte FM (1997) Biochimie 79:3-6
Eapen S, George L (1993) Plant Cell Tissue Organ Cult 35:151-156
Edwardson, J.R.(1974)Florida Agricultural Experiment Station Monograph,Series 4,225pp.
Enaksha RM, Wickermesinhe ERM, Arteca RN (1993) Plant Cell Tissue Organ Cult 35:181-193
Eva W (1999) Plant Cell Tissue Organ Cult 57:57-60
Falcone AM, Leva AR (1986) Rivista di Agricoltura Subtropicale e Tropicale 80(1)71-79
FAO stats (2001) www.fao.org
Feldmann KA, Marks MD (1987) Mol Gen Genet 208:1-9
Fellman CD, Read PE, Hosier MA (1987) Hort Sci 22:1197-1200
Filichkin SA, Gelvin SB (1993) Mol Microbiol 8:915-926
Fisher RA (1954) "Statistical Methods for research workers "12 edn, Oliver and Boyd, Edinburgh and London.
Fisk HJ, Dandekar AM (1993) Sci Hort 55:5-36
Fitch MMM (1991) Development of genetic transformation systems for papaya. Un published Ph.D. thesis, University of Hawaii.
Fitch MMM (1993) Plant Cell Tissue Organ Cult 32 :205-212
Fitch MMM (1995) Somatic embryogenesis in papaya. In: Biotechnology in Agriculture anf Forestry 30. Somatic embryogenesis and Synthetic seeds 1(ed Y.P.S. Bajaj), Springer Verlag Berlin heidelberg
Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL (1993) Plant Cell Rep 12(5): 245-249
Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1992) Biotech 10: 1466-1472
Fitch MMM, Mansherdt RM (1990) Plant Cell Rep 9:320-324.
Fitch MMM, Mansherdt RM, Gonsalved D, Slightom JL, Sanford JC (1990) Plant Cell Rep 9:189-194
Flath RA, Forrey RR (1977) J Ag and Food Chemistry 25(1):130-109
Fray RG, Grierson D (1993) Trends Genet 9:439-443
Frenkel MJ, Ward CW, Shukla DD (1989) J. Gen Virology 70: 2775-2783
Furutani SC, Nagao MA (1986) Hort Science 21:6
Furutani SC, Nagao MA (1987) Scientia Hortic 32:67-72
Galau GA, Jacobsen KS, DW Hughes (1990) Physiol Plant 81:280-288
Gamborg OL, Miller RA, Ojima K (1968) Exp Cell Res 50:151-158
Gasser C, Fraley RT (1989) Science 244:1293-1299

Gelvin SB (2000) In: Jones RL, Bohnert HJ, Delmer DP (eds) Ann Rev Plant Physiol Plant Mol Biol Annual Reviews, Palo Alto, California, 51:223-256
Gelvin SB, Liu CN (1994) In: Gelvin SB, Schilperoort RA (eds) Plant Molecular Biology Manual, Kluwer Academic Publishers, Dordrecht B4:1-3
Genga A, Allavena A (1991) Plant Cell Tissue Org Cult 27:189-196
Georges F, Saleem M, Cutler AJ (1990) Gene 91:159-165
Ghazi TD, Cheema HV, Nabors MW (1986) Plant Cell Rep 5:452-456
Gill R, Saxena PK (1992) Can J Bot 70:1186-1192
Goldfarb B, Howe GT, Bailey LM, Strauss SH, Zaerr JB (1991) Plant Cell Rep 10:156-160
Gong Z (1992) Bio techniques12:74-75
Gonsalves D (1998) Annual Rev Phytopatho 36:415-437
Gonzalez MV, Rey M, Tavazza R, LaMaifa S, Cuozzo L and Ancora G (1998) Hort Sci 33(4):749-750
Granier F, Durand Tardif M, Casse-Delbert F, Lecoq H, Robaglia C(1993) J. Gen Virol 74: 2737-2742
Griesbach RJ, Hammond J (1993) Acta Hortic 336:165-169
Guo Y, Liang H, Berns MW (1995) Physiol Plant 93:19-24
Gupta PK, Durzan D (1987) Bio/Tech 5:147-151
Habben JE, Larkins BA (1995) Curr opi Biotech 6:171-174
Haccius B (1978) Phytomorphology 21:103-107
Hanahan D (1985) In: DNA Cloning Volume 1,ed. D. Glover, IRL Press Ltd. 109-135
Hand DJ, Craig G, Takaki M, Kendrick RE (1992) Planta 156:457-460
Hari V, Siegel A, Rozek C, Timberlark WE (1979) Virology 92 : 568-571
Hari V (1981) Virology 112: 391-399
Hartweek LM, Lazzeri PA, Cui D, Collins GB, Williams EG (1988) In vitro Cell Dev Biol 24: 821-828
Hay JM, Fellowes AP, Timmerman GM (1989) Arch Virol 107: 111-122
Hazra S, Sathaye SS, Mascarenhas AF (1989) Bio/Technology 7:949-951
Heibert E, McDonald JG (1976) Virology 70: 144-150
Heibert E, Tremaine JH, Roland WP (1984) Phytopathology 74: 411-416
Herrera -Estrella A, Van Montagu M, Wang K (1990) Proc Natl Acad Sci USA 87:9534-9537
Herrera Estrella L, De Block M, Messens E, Hernalsteens JP, Van Montagu M, Schell J (1983) EMBO J 2:987-995
Hicks GS (1994) In vitro Cell Dev Biol 30p:10-15.
Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Plant J 6:271-282
Hinchee MAW, Corbin DR, Armstrong ChL, Fry JE, Sato SS, DeBoer DL, Peterson WL, Armstrong TA, Connorward DV, Layton JG, Horsch RB (1994) Plant Transformation In: Vasil IK, Thorpe TA (eds) Plant Cell and Tissue Culture, Kluwer Academic Publisher, Dordrecht, Netherlands, pp 231-270
Hoekema A, Huisman M, Molendijk L, van den Elzen P, Cornelissen B (1989) Bio/Technology 7:273-278
Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) Transgenic Res 2:208-218
Hooykaas PJJ, Beijersbergen AGM (1994) Ann Rev Phytopath 32:157-179

Horch RB, Fraley RT, Rogers SG, Saners PR, Lioyd A, Hoffman N (1984) Science 223:496-498
Horovitz S, Jimeenez H (1967) Agron Trop (Maracay) 17:323-326
Hossain M, Rahman SM, Islam R, Joarder OI (1993) Plant Cell Rep 13:99-102
Huang JC, Ye KN, Cheng G, B Li (1996). Acta Scientiarum Naturalium universitatis Sunyatseni 35(4):90-94
Huet H, Gal on A, Meir E, Lecoq H, Raccah B (1994) J Gen Virol 75: 1407-1414
Huetteman CA, Preece JE (1993) Plant Cell Tissue Organ Cult 33:105-119
Huttings H, Mosch WHM (1974) Neth J Plant Pathol 80: 19-27
Islam R, Rahman SM, Hossain M, Joarder OI (1993) Pakisthan Journal Of Botany 25(2):189-192
Islam R, Rahman SM, Hossain M, Joarder OI (1993) Plant Tissue Cult 3(1):47-50
Jagadish MN, Ward CW, Gough KH, Tulloch PA, Whittaker LA, Shukla DD (1991) J Gen Virol 72: 1543-1550
Janick J (1991) In: Rattray J (Ed) Biotechnology of plant fats and oils, Am Oil Chem Soc Champaign pp76-104
Janick J (1993) Act Hort 336: 207-215
Jefferson RA (1989) Nature 342:837-838
Jenes B, Morre H, Cao J, Zhang W, Wu R (1993) Techniques for gene transfer. In: Kung S, Wu R (eds) Trangenic plants, Academic Press, San Diego, Vol 1, pp 125-146
Jimenez H, Horovitz S (1958) CIA Venez Agron Trop 7:207-215
Joanin L, Brasileiro ACM, Leple JC, Pilate G, Cornu D (1993) Ann Sci For 50:325-336
Joersbo M, Brunstedt J (1990) Plant Cell Rep 9:207-210
Jordan M, Cortes I, Montenegro G (1982) Plant Sci Lett 28:321-326
Kaeppler HF, Gu W, Somers DA, Rhines HW, Cockburn AF (1990) Plant Cell Rep 9:415-418
Kameya T, Widholm J (1981) Pl. Sci lett 21:289-294
Kanyand M, Deasi AP, Prakash CS (1994) Plant Cell Rep 14(1):1-5
Kar S, Johnson TM, Nayak P, Sen SK (1996) Plant Cell Rep 16:32-37
Kawasaki ES (1990) in PCR protocols (MA Innis, DH Gelfannd, JJ Sninsky TJ White Eds) (San Diego, Academic Press).

Kerns HR, Barwale UB, Meyer MMJr, Wildholm JM (1986) Plant Cell Rep 5:140-143
Khan AA, Heit CE, Waters EC, Anolulu CC, Anderson L (1971). New York Agril. Exp sci General 1(9):1-12
Khuspe SS, Hendre RR, Mascerenhas AF, Jagannathan V (1980) Utilization of tissue culture to isolate interspecific hybrids in Carica papaya L. In: Rao PS, Heble MR and Chaddha Ms (eds) Plant Tissue Culture genetic manipulation and somatic hybridization of plant Cells . Bombay BARC pp 198-205
Kim MK, Somma HE, Bongarten BC and Merkle SA (1997) Plant Cell Rep 16:536-540
Kim MS, Schumann CM and Kloptenstein NB (1997) Plant Cell Tissue Organ Cult 48:45-52
Klee H, Horsch R, Rogers S (1987) Annu Rev Plant Physiol 38:467-486
Klein TM, Wolf ED, Wu R, Sanford JC (1987) Nature 327:70-73

Knight RJ (1980) Origin and world importance of tropical and subtropical fruitcrops. In: Tropical and Subtropical Fruits: Composition, properties and uses (S. Nagy and p.e. Shaw, eds)pp.-1-120. AVI publishing, Westport, Connecticut
Konar RN, Nataraja K (1965) Phytomorphology 15:132-137
Kong L, Yeung EC (1994) Plant Sci 104: 71-80
Kong L, Yeung EC (1995) Physiol Plant 43: 298-304
Kozai T (1991) Micropropagation under photoautotrophic conditions In: Debergh PC, RH Zimmerman (eds) Micropropagation: Technology and Application. Kluwer Academic Publisher, Dordrecht pp 447-469
Krishnamurthy KV, Suhasini K, Sagare AP, Meixner M, de Kalhen A, Pickardt T, Schieder O (2000) Plant Cell Rep 19: 235-240
Kung SD (1984) Critical Rev Plant Sci 1:227-267
Kung SD (1993) From hybrid plants to transgenic plants. In: Kung SD,Wu R (eds) Transgenic plants, Academic Press, San Diego, Vol 1, pp1-10
Kuwahara YC, Orika OE, Marchilia DOS (1999) Sci Agric 56:9-12
Kyauk H, Hopper NW, Brigham RD (1995) Environ. Exp Bot 35: 345-351
Kysely W, HJ Jacobsen (1990) Plant Cell Tissue Organ Cult 20:7-14
Lad BI, Jayasankar S, Pliego-Alfaro F, Moon PA, Litz RE (1997) In Vitro Cell Dev Biol 33P: 253
Lai CC,Yu TA,Yeh SD,Yang JS (1998) Plant Cell Tissue Organ Cult 53:221-225
Lakshmisita G, Muralidhar Rao M (1997) Direct somatic embryogenesis from immature
Lange AH (1961) Bot Gaz 122(4):305-311
Lawson C, Kaniewski W, Haley L, Rozman R, Newell C, Sanders P, Tumer N (1990) Bio/ Technology 8:127-134
Lazzeri PA, Hildebrand DF,Collins GB (1987b) Plant Cell Tissue Org Cult 10:209-220
Leblay C, Chevreau E, Raboin LM (1991) Plant Cell Tissue Organ Cult 25:99-105
Lehrach H, Diamond D, Wozney JM, Boedtker H (1977) Biochemistry16:4743-4751
Leifert C, Murphy KP, Lumsden PJ (1995) Crit Rev Plant Sci 14(2):83-109
Leonel Sarita, Rodrigues JD (1999) Sci. Agric. 56: 111-115.
Linsmaier EM, Skoog FA (1965) Physiol Plant 18:100-127
Litz RE (1984) Papaya In: Sharp W, Evans D, Ammirato P, Yamada Y(eds) Handbook of plant cell cultures Vol 2 Crop species. Macmillan London, pp 349-368
Litz RE (1986) Papaya (Carica papaya L.) In: Bajaj YPS(ed) Biotechnology in agriculture and forestry Vol 1 Trees 1. Springer, Berlin Heidelberg, Newyork, pp 220-232
Litz RE (1986) Papaya. In: Biotechnology in Agriculture anf Forestry 1. Trees 1 (ed Y.P.S. Bajaj), Springer Verlag Berlin heidelberg agriculture and forestry, vol II, Springer, Berlin heidelberg, New York. pp 85-111
Litz RE, Conover RA (1962) Hort Sci 13:241
Litz RE, Conover RA (1977) Proc Fla State Hortic Soc 90:245-246
Litz RE, Conover RA (1978a) Hort Sci 13:241-242
Litz RE, Conover RA (1978b) Proc Fla State Hortic Soc 91:182-184
Litz RE, Conover RA (1979) Proc Fla State Hortic Soc 92:180-182
Litz RE, Conover RA (1980) Hort science 15:733-735
Litz RE, Conover RA (1981b) Z Pflanzenphysiol 104:285-288
Litz RE, Conover RA (1982) Plant Sci Lett 26: 153-158

Litz RE, Conover RA (1983) Ann Bot. 51;683-686
Litz RE, Conover RE (1981a) J Am Soc Hortic Sci 106:792-794
Litz RE, Gray DJ (1995) World Jof Microbiol and Biotech 11:416-425
Litz RE, Moore GA, Srinivasan C (1985) Hort Rev 7:157-200
Litz RE, O'Hair SK, Conover RA (1983) Sci Horti 19:287-293
Lu CY (1993) In Vitro Cell Dev Biol 29P :92-96
Luo ZX, Wu R (1988) Plant Mol Biol Rep 6:165-174
Lutchke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA (1987) EMBO J 6:43-46
MacDonald RJ, Swift GH, Przybyla AE, Chirgwin JM (1987) Methods Enzymol 152:219-227
Magioli C, Rocha APM, Oliveira DE, Mansum E (1998) Plant Cell Rep 17 (8):661-663
Magnavel C, Noir M, Vermeil JL, Blats A, Hut C, Grosdemange F, Buele T, Buffard Morel J (1997) J Plant Physiol 150: 719-728
Mahon RE, Bateson MF, Chamberlain DA, Higgins CM, Drew RA, Dale JL (1996) Aust J Plant Physiol 23:679-685
Malik KA , Alikhan ST, PK Saxena (1993) Annals of Botany 72:629-637
Mallik KA, Saxena PK (1992a) Plant Cell Rep 11:163-168
Manshart RM (1999) ' UH Rainbow' papaya. University of Hawaii College of Tropical Agriculture and Human Resources, New Plants for Hawaii-1. Honolulu: University of Hwaii College of Tropical of Tropical Agriculture and Human Resources.
Mansherdt RM, Wenslaff TF (1989a) J. Am Soc Hortic Sci 114:684-689
Mansherdt RM, Wenslaff TF (1989b) J. Am Soc Hortic Sci 114:689-694
Marcotrigiano M (1990) Genetic mosaics and chimeras: implications in biotechnology.In: Bajaj YPS (eds) Somaclonal variation in crop improvement I. Biotechnology in agriculure and Forestry, Vol II, Springer, Berlin Heidelberg, New York. Pp 85-111.
Marunda CT (1990) ACIAR Proceedings Series No 28.
Mason HS, Man-Kit Lam D, Arntzen CJ (1992) Proc Natl Acad Sci USA 89:11745-11749
Massimo MS, Murch SJ, Slimmon TY, Krishnaraj S, Saxena PK, (1995) Morphoregulatory role of thidiazuron: morphogenesis of root outgrowths in thidiazuron-treated geranium (Pelargonium x hortorum Bailey) Plant Cell Rep 15:205-211
Mathews H, Schopke C, Carcamo R, Chavarriaga P, Faquet C, Beachy RN (1993) Plant Cell Rep 12:328-333
Mathias RJ, Boyd LA (1986) Plant Sci 46:217-223
Mathias RJ, Mukasa C (1987) Plant Cell Rep 6:454-457
Matthysse AG, Thomas DL, White AR (1995) J Bacteriol 177:1076-1081
Mayer AM, Poljakoft-Mayber A (1979) The germination of seeds 2nd edition Pergamon press, NewYork USA.
McClelland MT, Smith MAL (1990) Hort Sci 25:797-800
McElroy D, Brettel RIS (1994) Trends Biotechnol 12:62-68
McGranahan GH, leslie CH, Uratsu SL Martin LA, Dandekar AM (1988) Biotechnology 6:800-804

McKently AH, Moore GA, Dooster H, Niedz RP (1995) Plant Cell Rep 14:699-703
McKersie BD, Brown DCW (1996) Seed Science Research 6:109-126.
Medora RS, Mell GP, Bilderback DE (1984) Zeitschriftfur pflanzenphysiologie 114(2):179-185
Mehdi AA, Hogan L (1976) Hort Sci 11:311
Mehdi AA, Hogan L (1979) Hort Sci (Abs) 14:46
Merkle SA, Parrott WA, Flinn BS (1995) In: Thorpe TA (ed) In vitro embryogenesis in plants, Kluwer Academic Publishers, Dordrecht, pp 155-203
Merkle SA, Parrott WA, Williams EG (1990). Application of somatic embryogenesis and embryo cloning. In: Plant Tissue Culture: Applications and Limitations S.S.Bhojwani (eds) Elsevier, Amsterdam pp67-101
Meyerowitz EM (1987) Plant Mol. Biol. Rep 5 (1):242-250
Mhaske VB, Hazra S (1994) In vitro Cell Dev Biol 30P:113-116
Miller RM, Drew RA (1990) Plant Cell Tissue Organ Cult 21:39-44
Mok MC, Mok DWS, Armstrong DJ (1980) Plant Physiol 65 (suppl):24 (Abstr)
Mondal M, Gupta S, Mukherjee BB (1990) Plant Cell Rep 8:609-612
Mondal M, Gupta S, Mukherjee BB (1994) Plant Cell Rep 13:390-393
Monmarson S, Nicole michaux F, Teisson C (1995) J. Hort Sci 70:57-64
Moore GA, Litz RE (1984) J. Am Soc Hortic Sci 109:213-218
Morton JF (1977) Major medicinal plants. CC Thomas, Springfield, IL
Murai N, Suton DW, Murray MG, Slighton JL, Merlo DJ, Reichart NA, Sengupta Gopalan C, Stock CA, Barker RF, Kemp JD, Hall TC (1983) Science 22: 476-482
Murashige T, Skoog F (1962) Physiol Plant 15:473-497
Murthy BNS, Jerrin V, RanaSingh P, Fltcher RA, Saxena PK (1996) Plant Growth Regulator 19(3):233-240
Murthy BNS, Saxena PK (1998) Plant Cell Rep 17:469-475
Murthy GVK, Natarajan CP (1982) Papaya a commercial crop for the production of papain and pectin. P 436-446. In Atal CK and Kapur BM (eds), Cultivation and Utilization of medicinal plants. CSIR, Jammu Tawi.
Muthukrishnan –CR, Irulappan I (1987) PAPAYA Ed by T.K. Bose and S.K.Mitra. Naya Prokash, 206, Bidhan Sarani Calcutta Six
Nagao MA, Furutani SC (1986) Hort Sci 21:1439-1440
Nagaveni HC, Srimathi RA (1980) The Indian Forester 106:792-800
Naidu CV, Rajendrudu G, Swamy PM (1999) Seed Sci and Technology 27:885-892
Nam J, Matthysse AG, Gelvin SB (1997) Plant Cell 9:317-333
Narciso JOY, Futsuhara K, Hattori , Wada T (1997) Japan J of Crop Science 66:67-75
Neuhaus G, Spangenberg G (1990) Physiol Plant 79:213-217
Nickle TC, Yeung EC (1994) In vitro Cell Dev Biol 30P:96-103
Obeidy AA, Smith MAL (1993) Hort Sci 28(3):213-215
Ohta Y (1986) Proc Natl Acad Sci USA 83:715-719
O'Neal J (2001) http://ceprap.ucdavis.edu/Transformation/transform1.htm
Owen HR, Wengard D, Miller AR (1991) Plant Cell Rep 10:583-586
Ozias-Akins P (1989) Plant Cell Rep 8:217-218
Ozias-Akins P, Singsit C, Branch WD (1992b) J. Plant Physiol 140:207-212
Ozkan S, Sevimay CS, Yildiz M, Sancal C, Ozgen M (1996) Plant Cell Rep 16:200-203

Page YM, Staden JY (1985) In vitro propagation of Dierama latifolium. Hort Sci 20: 1049-1050

Pancholi N, Wetten A, Caligari PDS (1995) In vitro Cell Dev Biol Plant 31:127-130

Pang Z, Sanford JC (1988) J. Am Soc Hortic Sci 113:287-291

Parrott WA, RE Durham, Bailey MA (1995) Somatic embryogenesis in legumes. In: Biotechnology in agriculture and Forestry. Vol 31, somatic embryogenesis and synthetic seed II (Ed YPS Bajaj). Springer-Verlag Berlin Heidelberg,pp 199-227

Pauls KP (1995) Biotech Advances 13:673-693

Perez RM, Galiana AM, Navarro L, Duran-vila N (1998) J. of Hort Sci and biotech 73: 796-802.

Perlak FJ, Deaton RW, Armstrong TA, Fuchs RL, Sims SR, Green plate JT, Fischhoff DA (1990) Bio/Technology 8:939-943

Pernezny K, Litz RE (1993) Series of pant pathology Dept university of Florida, Co-operative extension service. Institute of food and Agricultural Services pp-35

Phadnis NA, Budrakkar ND, Kaulgud SN (1970) Poona Agril Coll Mag 60:101-104

Philip S, Bindu MR, Anandraj M, Sarma YR (1997) in Recent advances on Biotechnology. Applications of Plant Tissue and Cell culture, Oxford IBH Publications. New Delhi, India, pp-269-273

Pierik RLM (1987) In In vitro culture of higher plants M Nijhoft Publications Dordrecht 183-230

Pirone TP (1991) Sem Virol 2: 81-87

Pirone TP, Thornbury DW (1983) Phytopathology 73: 411-416

Podwysznska M, Olszeweski T (1995) Sci Hort 64:77-84

Poirier Y, Nawrath C, Somerville C (1995) Bio/Technology 13:142-150

Pollock K, Barfield DG, Shields R (1983) Plant Cell Rep 2:36-39

Potrykus I (1995) In: Potrykus I and Spangenberg G (Eds) Gene transfer to plants, Springer Verlag, Berlin, pp 5-9

Prasad JS, Raj Kumar, Mishra M, Kumar R, Singh AK, Prasad U (1996) Hort Sci 31(7):1187-1189

Predieri S, Fasolo FMF, Passey AJ, James DJ (1989) J. of Horti Sci 64:553-559

Purcifull de, Edwardson J, Hiebert E, Gonsalves D (1985) Papaya Ringspot Virus (revised) No 84 In: Descriptions of palnt viruses. Common w Mycol Ins/Assoc Appl Biol kew Surrey

Purseglove JW (1968) Papaya. In: Tropical crops Vol 41 Dicotyledons. John Wiley, NewYork

Purseglove JW (1974) Piper nigrum. In: Tropical crops: Dicotyledons, Longman Group Ltd, pp 441-450

Quemada H, L'Hostis B, Gonsalves D, Reardon IM, Heinrikson R, Hielbert EL, Sieu LC, Slightom JL (1990) J. Gen Virol 71:203-210

Quemada H, L'Hostis B, Gonsalves D, Reardon IM, Heinrikson R, Hiebert EL, Siue LC, Slightom JL, (1990) J Gen Viol.71:203-210

Ragahavan V (1976) Embryogenesis in angiosperms: A developmental and experimental study, Cambridge University Press, Cambridge.

Rajasekaran K, Vini J, Mullins MG (1982) Planta 154:139-144

Rajeevan MS, Pandey RM (1983) Acta Horticulturae 131-137

Rajeevan MS, Pandey RM (1986) Plant Cell Tissue Organ Cult. 6:181-188
Rangaswamy NS (1986) Proc. Ind. Acad Sci (Plant Sci) 96(4):247-271
Ravindran PN, Nair MK, Muneer MK (1985) J. plantation Crops 13:132-158
Reed CF (1976). Information summaries on 1000 economic plants. Type scripts submitted to USDA--www.hort.purdue.edu/newcrop/duke_energy/refa-f.html.
Relf D (1997) Extension Specialist, Environmental Horticulture, Virginia polytechnoque and state university, March 3, 1997 Seed Germination and Soil Temperature Contact http://www.ext.vt.edu/departments/envirohort/articles2/sdgrmtmp.html
Reuhs BL, Kim JS, Matthysse AG (1997) J Bacteriol 179:5372-5379
Reuveni O, Shlesinger DR, Lavi U (1990) Plant Cell Tissue Org Cult 20:41-46
Riechmann JL, Lain S, Garcia JA (1990) Virology 177:710-716
Riechmann JL, LainS, GarciaJA (1991) Virology 185:544-552
Riechmann JL, LainS, GarciaJA (1992) J GenVirol 73: 1-16
Ritchie SW, Lui CN, Sellmar JC, Kononowicz H, Hodges TK, Gelvin SB (1993) Transgenic Res 2:252-265
Robagalia CT, M Tronchet, Boudazin G, Astier-Manifacier S, Casse Deelbart F (1989) J Gen Virology 70:935-937
Roberts DR, Suton BC, Flinn BS (1990) Can J Bot 68:1086-1090
Robinsons KEP, Firoozabady E (1993) Sci Hortic 55:83-99
Roustan JP, Latchs A, Fallot J (1990) Plant Sci 67:89-95
Saeed NA, Zafar Y, Malik KA (1997) Plant Cell Tissue Org Cult 51:201-207
Sagare AP, K Suhasini, Krishnamurthy KV (1993) Plant Cell Rep 12:652-655
Sagare AP, Suhasini K, Krishnamurthy KV (1993) Plant Cell Rep 12:652-655.
Saloman R (1989) Res Virol 104: 453-455
Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning: a laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
Sanford JC (1988) Trends Biotechnol 6:299-302
Sangwan RS, Ducrocq C, Sangwan -Norreel B (1993) Plant Sci 95:99-115
Satrija F, Nansen P, Bjorn H, Murtini S, He S (1994). J of Helminthology 68(4): 343-346
Schenk RU, Hilderbrandt AC (1972) Can J Bot 50:199-204
Schieder O (2000) Plant Cell Rep 19:235-240
Schnabelranch LS, Sink KC (1979) Hort Sci 14:607-608
Schroeden KR, Stimart DP (1999) Hort Sci 34(4):736-739
Seeber G, Agpaoa A (1976). Forest Tree Seeds. In: Manual of Forest tree seed in Zimbabwe - a review of methods and results Zimbabwe Bull For. Res. No. 8. For Commission. Salisburry.
seeds of Rosewood (Dalbergia latifolia Roxb), in Trends in Plant Tissue Culture and Biotechnology, edited by LK Pareek (Agro botanical Publisher, Bikaner, India) 271
Sen SK, Gunthi P (1977) Food Farming Agric 9:144-146
Shankarraja NS, Sulikeri GS (1993) J. Plantation Crops 21:116- 117
Shaquiang Ke, Skirvin RM, Mcpheeters KD, Otterbachet AG, Galletta G (1985) Hort Sci 20:1047-1049
Sharma AK, Sharma A (1980) Chromosome techniques, theory and practice. Butterworths, London, pp 71-81

Sharma HC, Singh SK, Goswami AM, Singh SP (1999).Antibiotics and their role in in vitro culture establishment of field grown papaya. In : Plant Tissue Culture and Biotechnology emerging trend (ed) Kavikishore PB, University press (india Ltd, Hyderabad-29,151-155

Sharma VC, Ogbeide IN (1982) Energy 7(10):871-873

Sharp WR, Sondahl MR, Caldas LS, Maraffa SB (1980) Hort Rev 2:268-310

Shaw CH (1988) Plant Molecular Biology: A practical approach pp.135

Shekhawat NS, Rathore TS, Singh RP, Deora NS, Rao SR (1993) Plant Growth Regulators 12:273-280

Shlesinger D, Reuveni O, Lavi U (1987) Acta Hort (ISHS) 212:570

Shukla DD Ward CW (1988) J Gen Virology 69: 2703-2710

Shukla DD, Strike PM, Tracy SL, Gough KH, Ward CW (1988) J Gen Virology 69:1497-1508

Shukla DD, Tribbick G, Mason TJ, Hewish DR, Geysen HM, Ward CW (1989) Proc. Natl. Acad. Sci USA 86: 8192-8196

Siaw MFE, Shahabuddin M, Ballard S, Shaw JG, Roads RE (1985) Virology 142:134-143

Siemens J, Schieder O (1996) Plant Tissue Cult Biotech 2: 66-75

Simons JN (1976) Phytopathology 66: 652-654

Singh RN (1964) Indian J. Hort 21 (2):148-154

Singh SP, Dabhiya SS (1982) Fmr and Parliament 17:15-16

Slighton JL (1991) Gene 100:251-255

Smith EF,Townsend CO (1907) Science 25:671-673

Smith RH, Hood EE (1995) Crop Science 35:301-309

Smith TJ (1994) Biotech Advances 12:679-686

Songstad DD, Armstrong CL, Peterson WL (1991) Plant Cell Rep 9:699-702

Songstad DD, Somers DA, Griesbach RJ (1995) Plant Cell Tissue Organ Cult 40:1-15

Sookmark S, Tai EA (1975) Acta Hortic 49:85-90

Srivatanukul M, Park SH, Sanders JR, Salas MG, Smith RH (2000) Plant Cell Rep 19(12):1165-1170

Steward FC (1958) Amer J Bot 45:709-713

Steward FC, Mapes MO, Kent AE, Holsten RD (1964) Science 163:20-27

Stomp AM (1992) In: GUS protocols: using the GUS gene as a reporter of gene expression, Academic Press, California, pp 103-112

Strickland SG, Nichol JW, McCall CM, Stuart DA (1987) Plant Sci 48:113-121

Stuart DA, MK Redenbaugh MK (1987) Use of somatic embryogenesisfor the regeneration of plants. In: Biotechnology in agricultural chemistry (eds)Le Baron HM, Mumma RO, Honey cutt RC and Duesing JH. ACS symposium series 334. Pp86-96

Suhasini K, Sagare AP, Krishnamurthy KV (1994) Plant Sci 102:189-194.

Suhasini K, Sagare AP, Krishnamurty KV (1996) In vitro Cell Dev Biol 32P: 6-10

Sujatha M, Reddy TP (1998) Plant Cell Rep 17:561-566

Sukhla DD, Frenkel MJ, Ward CW (1991) Can J Plant Pathol 13: 178-191

Sul IW, Korban SS (1998) J. Hort Sci Biotech 73(6):822-827

Taji AM, Williams RR (1989) Plant Cell Tissue Organ Cult 16:61-66

Tennant PF, Gonsalves C, Ling KS, Fitch M, Mansherdt R, Slightom JL, Gonsalves D (1994) Phytopathology 84:1359-1366

Tennnant P, Fermin G, Fitch MM, Mansherdt RM, Slightom JL, Gonsalves D (2001) Euro J Plant Pathlogy (In press)

Tepfer D (1984) Cell 37:959-967

Thole V, Dalmay T, Burgyan J, Balazs E (1993) Gene 125: 149-156

Thorpe TA (1982a) Carbohydrate utilization and matabolism. In: Bonga JM and Durzan DJ (eds) Tissue culture in Forestry. Martinus Nijhoff Publishers London pp 325-368

Thorpe TA (1983) Beltsville symposium Agric Res 7:285-303

Thorpe TA (1988) In vitro somatic embryogenesis, In: ISI Atlas of science Animal and Plant Sciences pp 81-88

Thorpe TA (1988) In vitro somatic embryogenesis. In: ISI Atlas of Science. Animal and Plant Science pp 81-88

Thorpe TA (1994) Morphogenesis and regeneration In: Plant Cell and Tissue Culture (EDS) I.K. Vasil and T.A.Thorpe. Kluwer Academic Publishers, Dordrecht, Netherlands, pp 17-36

Tissert B, Esan EB, Murashige T (1979) Hort Rev 1:1-78

Tort N (1996) J. Agro & Crop Sci. 176: 217-221.

Tran Thanh Van KM (1981) Ann Rev Plant Physiol. 32:219-311

Trigiano RN, Beaty RM, Grahanan ET (1988) Plant Cell Rep 7:148-150

Tsay HS, Su CY (1985) Plant Cell Rep 4:28-30

Tseng Mei Tsang (1991) Guoli Taiwan Daxue Nongxueyuan Yanjiu Baogao 31:30-39

Tuleke W, McGranahan G (1985) Plant Sci 40: 57-63

TurpenT (1989) J Gen Virol 70: 1951-1960.

Van der Sman AJM, Blom CWPM, Van de Steeg HM (1992) Canadian Journal of Botany 70(2):392-400

Van Nieuwkerk JP, Zimmerman RH, Fordham I (1986) Hort Sci 21:516-518

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, RochaSosa M (1990) Mol Gen Genet 220:245-250

Vasil IK, Vasil V (1986) Regeneration in cereal and other grass species. In: Cell culture and somatic cell genetics of plants vol 3. IK Vasil (ed) Academic Press, New york pp 121-150

Vega de Rojas R, Kitto SL (1991) J. AM Soc Hortic Sci 116:747-752

Veluthambi K, Ream W, Gelvin SB (1988) J Bacteriol 170:1523-1532

Vogel AM, Das A (1992) J Bacteriol 174:303-308

Walden R, Schell J (1990) Eur J Biochem 192:563-576

Walden R, Wingender R (1995) Trends Biotechnol 13:324-331

Wang CH, Bau HJ, Yeh SD (1994) Phytopathology 84:1205-1210

Wang HL, Yeh SD, Chiu RJ, Gonsalves D (1987) Plant Dis 71:491-497

Ward CW, Shukla DD (1991) Intervirology 32: 269-296

Webb KJ, Morris P (1992) Methodologies of plant transformation. In: Gatehouse AMR, Hilder VA, Boulter D (eds) Pant genetic manipulation for crop protec6tion. CAB In, Wallingford, Oxon, UK, pp7-43.

Webber N, Taylor DC, Underhill EW (1992) Adv Biochem Engn Biotechnol 45: 99-131

White PR (1963) A hand book of plant and animal Tissue culture. Jaques Cattel, Lancaster, Pennsylvanis
White PR (1963) The cultivation of animal and plant cells (2nd ed). 2 Ronald Press,
Williams EG, Maheshwaran G (1986) Ann Bot 57:443-462
Wilmink A, van de van BCE, Dons HJM (1995) Plant Mol Biol 28:949-955
Winner MD (1988) Pant Cell Tissue Org Cult 12:306
Wright MS, Ward DV, Hinchee MA, Carnes MG, Kaufman RJ (1987) Plant Cell Rep 6: 83-89
www.apsnet.org/online/feature/papaya/Top.html
Xy LI, Huang F (1996) In Vitro Cell Dev Biol 32P: 129-132
Yahiro M, Yoshitaka H (1982) Nettai Nogyo 26: 63-67
Yamamoto H, Tabata M (1989) Plant Cell Rep 8:251-254
Yamamoto H, Tanaka S, Fukui H and Tabata M (1986) Plant Cell Rep 5:269-272
Yang JS, Ye CA (1992) Bot Bull Acad Sin 33:375-381
Yang JS, Yu TA, Cheng YH, Yeh SD (1996) Plant Cell Rep 15:549-564
Yates IE, Wood BW (1989) J Amer Soc Hort Sci 114(6):1025-1029
Ye KN, Ma L, Li BJ (1991) Genet manipulation Plants 7:60-66
Ye K-N, Ma L, Li BJ (1991) Genet Manipulation Plants 7:60-66
Yeh SD, Gonsalves D (1984) Phytopathology 74:1086-1091
Yeh SD, Gonsalves D (1985) Virology 143:260-271
Yeh SD, Gonsalves D, Wang HL, Namba R, Chiu RJ (1988) Plant Dis 72:375-380
Yeh SD, Jan FJ, Chiang CH, Doong TJ, Chen MC, Chung PH, Bau HJ (1992) J Gen Virol 73:2531-2541
Yie S, Liaw SI (1977) In vitro 13:564-568
Yip NK, Yang SF (1986) Sci. Agric 56:9-12
Yu TA, Yeh SD, Yang JS (2001) Bot Bull Acad Sin 42:281-286
Zambryski PC (1992) Annu Rev Plant Physiol Plant Mol Biol 43:465-490
ZaradSS, Hossny YA, El-Bagoury HM (I997) Egypt J Physiol Sci 21:147-159
Zhong H, Sun B, Warentin D, Zhang S, Wu R, Wu T, Sticklen MB (1996) Plant Physiol 110:1097-1107
Zhou GY, Huang J, Chen S (1988) Sci Agric Sinica 21:1-6
Zhou GY, Weng J, Zeng Y, Huang J, Qian S, Liu G (1983) Meth Enzymol 101:433-481
Zijian Li, Jarret RL, Pittman RN, Demshi JW (1994) In vitro cell Dev Biol 30P:187-191
Zimmerman JL (1993) Plant Cell 5:1411-1423
Zupan JR, Zambryski P (1995) Plant Physiol 107:1041-1047

AUTHOR'S PUBLICATION

RESEARCH WORK PUBLISHED

5. **Bhattacharya J.** and S.S.Khuspe (2001) *In vitro* and *in vivo* germination of papaya (*Carica papaya* L.) seeds. **Scientia Horticulturae** 91: 39-49.
6. **Bhattacharya J**., S.S.Khuspe, N.N Renukdas and S.K.Rawal (2002) Somatic embryogenesis and plant regeneration from immature embryo explant of papaya cv. Washington and Honey dew **Indian Journal of Experimental Biology** 40: 624-627
7. **Bhattacharya J.** and S.S.Khuspe (2001) 2,4,5-T induced somatic embryogenesis in papaya. **Journal of applied horticulture (**accepted for publication).
8. **Bhattacharya J.,** S.S.Khuspe, N.N Renukdas and S.K.Rawal (2002). Multiple shoot induction and plant regeneration from immature embryo axes of papaya. (Manuscript communicated).

PAPER PRESENTED IN INTERNATIONAL CONFERENCES / SYMPOSIA

5. **Bhattacharya J.,** N.N. Renukdas and S.S.Khuspe **(2001)** Multiple shoot formation and plant let regeneration from immature embryo axis of papaya **(***Carica papaya* L.). In: 2nd International symposium of biotechnology of tropical and subtropical species, Institute of Botany, Academia Sinica, Taipei, Taiwan, 5-9 November.
6. **Bhattacharya J.** and S.S.Khuspe **(2001)** High frequency embryo induction in Papaya (*Carica papaya* L.) using 2,4,5-T. In: 4th International Plant Tissue Culture Conference at Dhaka, Bangladesh 1-3 November, 2001.
7. S.S.Khuspe, ML Mohan, S.K.Rawal and **J. Bhattacharya (2000):** Isolation and expression of PRSV (Papaya Ringspot Virus) coat protein gene. In: 3rd International Crop Science Congress 2000, Hamburgh, Germany, 17-22nd August.
8. **Bhattacharya J.** and S.S.Khuspe **(1999)** Direct somatic embryogenesis from immature zygotic embryos of papaya (*Carica papaya* L.). In: International Conference on Life Sciences in the next millenium, School of Life Sciences, University of Hyderabad, India, Dec 11-14.

PAPER PRESENTED IN NATIONAL CONFERENCES / SYMPOSIA

5. **Bhattacharya J.** and S.S.Khuspe (2001) Proliferative somatic embryogenesis from zygotic embryos of papaya (Carica papaya L.). In: 3rd National symposium on biochemical engn and Biotechnology, Biohorizon 2001,IIT, Delhi,23rd-24th February.
6. **Bhattacharya J.** and S.S.Khuspe (2001) 2,4,5-T induced somatic embryogenesis in papaya presented at 88th Indian Science Congress held in Delhi. IARI, Delhi from January 3 –7.
7. **Bhattacharya J**. and S.S.Khuspe (2000) Effect of seed type and growth regulators on in vitro seed germination of papaya (*Carica papaya* L.) seeds. Basic and applied aspect of Plant and microbial biotechnology5. Presented at Dept of Botany, Modern College, Feb 4-5th, 2000, Pune-5.

1) **Bhattacharya J.** and S.S.Khuspe (2000) *In vitro* regeneration of papaya through somatic embryogenesis. In: 87th Indian Science Congress held in Pune University, Pune from January 3 –7.