

**MICROPROPAGATION OF  
DATE PALM (*Phoenix dactylifera* L.) AND  
PAPAYA (*Carica papaya* L.)**

**BY**

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**PREFACE**

I hereby certify that the research work reported in this dissertation is the result of my own original investigation, except where acknowledged.



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I certify that the above statement is correct



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Supervisor

## **PUBLICATIONS FROM THIS THESIS**

McCUBBIN, M.J., VAN STADEN, J. and ZAID, A. (2000) A southern African survey conducted for off-types on date palms produced using somatic embryogenesis. Proceedings of the Date Palm International Symposium, Windhoek, Namibia, pp 68-72

McCUBBIN, M.J., ZAID, A. and VAN STADEN, J. (2000) The effects of sterilizing agents, plant growth regulators and explant type on somatic embryogenesis in date palm (*Phoenix dactylifera* L.) Proceedings of the Date Palm International Symposium, Windhoek, Namibia, pp 41-45

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## ABSTRACT

Date palms (*Phoenix dactylifera* L.) and papayas (*Carica papaya* L.) are two commercially important plantation crops. Their economic potential in South Africa and worldwide is increasing. However, due to disease, pests and socio-economic reasons, planting material is in short supply. Micropropagation provides a method for rapidly propagating selected superior cultivars for commercial and environmental interests.

A satisfactory process for the regeneration of elite cultivars should result in individuals phenotypically and genetically identical to the explant from which they were derived. However, due to somaclonal variation generated during *in vitro* culture, the true-to-typeness is questionable. For this reason a southern African survey for off-types on date palms produced using somatic embryogenesis was conducted. Plant growth variations such as leaf variegation, seedless fruit, broad leaves, compact growth habit and parthenocarpic fruit were recorded and possible explanations for each phenomenon given.

Factors influencing the date palm initiation process such as decontaminating agents, plant growth regulators, explant type and nurse cultures were investigated. A double decontamination process with 2.6% and 1.3% sodium hypochlorite was most effective at reducing contamination. Alternative plant growth regulators, TIBA and NAA were ineffective as a substitute to 2,4-D for somatic embryogenesis. The size of the explant and "nurse cultures" played an important role in explant growth and initiating callogenesis. A "nurse culture" reduced the time in culture significantly. The problem areas in the three commercial tissue culture techniques used for date palms were outlined.

In the second part of the study, factors influencing initiation, multiplication and rooting of papaya were determined. Presoaking with antibiotic, Rifampicin, and various fungicides had a positive effect on decontaminating papaya explants, while Bronocide™ had little effect. Various methods and materials were used to optimize papaya multiplication and rooting *in vitro*. The growth and multiplication of papaya was optimal at 50 g l<sup>-1</sup> sucrose. Gelling agent, Gelrite, increased multiplication rates

significantly but had a negative effect on overall growth causing plants to become vitrified. The addition of activated charcoal reduced vitrification but also reduced multiplication rate. Activated charcoal greatly improved overall growth of papaya and reduced leaf senescence. No vitrification was observed in multiplying papaya cultures where agar and Gelrite combinations were used, but multiplication rate was reduced compared to cultures grown on Gelrite alone. Callus removal from the bases of papaya at subculturing reduced multiplication rate and influenced elongation, growth and leaf senescence.

Lower concentrations of agar and Gelrite improved rooting percentages, but did not provide good support. Damaged roots and lower rooting percentages were observed on plantlets treated with IBA for four weeks compared to those exposed for only two days. A one hour pulse with a higher concentration ( $5 \text{ mg l}^{-1}$ ) of IBA greatly improved rooting percentage and further eliminated a second subculture onto an IBA-free medium after two days. Good, strong roots with root hairs were produced on vermiculite medium containing equal volumes of DS salts and vitamins. Modified lids with cotton-wool plugs also reduced leaf abscission.

*In vitro* grafting using stericrepe proved impractical, while grafting *in vitro* unrooted papaya plants onto *ex vitro* seedlings was more successful, using wedge and slant grafts. Grafts sealed with pegs and Parafilm™ were less effective.

## ABBREVIATIONS

2,4-D	2,4 -Dichlorophenoxyacetic acid
IBA	Indole-3-butyric acid
NAA	1- Naphthaleneacetic acid
BA	N <sup>6</sup> - Benzyladenine
iP	6-( <del>isopenteny</del> Dimethylallylamino) purine or N <sup>6</sup> -[2-Isopentenyl]adenine
AC	Activated charcoal
DS	Drew and Smith medium (1986)
M&S	Murashige & Skoog medium (1962)
Kinetin	N <sup>6</sup> - furfuryl adenine
PGR's	Plant growth regulators
TIBA	2,3,5- Triiodobenzoic acid
DF	DeFossard's modified medium
IAA	Indole-3-acetic acid
PVP	Polyvinylpyrrolidone
NOA	Naphthoxyacetic acid
dicamba	3,6-dichloro-2-methoxybenzoic acid
picloram	4-amino-3,5,6-trichloropicolinic acid
IAN	indole acetonitrile
STS	Silver thiosulphate

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## CHAPTER ONE

### LITERATURE REVIEW OF DATE PALM MICROPROPAGATION

#### 1.1 Introduction

The date palm (*Phoenix dactylifera* L.) ( $2n=36$ ) is one of the most important members of the Palmae (Arecaceae) family. There are 11 other species in the genus *Phoenix*, most of which are used as ornamentals. All species of *Phoenix* are dioecious with male and female flowers produced in clusters on separate palms in the axils of the leaves from the previous year's growth.

The date palm is subtropical in origin and cultivation. The exact centre of origin is unknown, but the earliest records (3000 B.C.) suggest Mesopotamia (Iraq) as a possible site (BRANTON and BLAKE, 1989). Date palms (Fig. 1A) today are grown in North and South America, Africa, Australia and the Middle East.

For many years date palms have been of immense value in maintaining and enhancing the quality of human life and has great socio-economic importance for Middle East inhabitants and elsewhere (POPENOE, 1973). The date palm is one of the most important cash crops in several subtropical countries where it represents a staple food for millions of people and a main source of income for the national economy (REYNOLDS, 1985). The fruit is highly nutritious and even from early times Roman writer Pliny said: "They are so remarkably luscious that there would be no end to eating them", while Persian poets render: "Dates and fish, are an Emperor's dish".

However, despite the importance of the date palm it is also plagued by problems. Date palm plantations in North Africa are currently in danger of being completely destroyed within a few years by a vascular fusariosis (Bayoud disease) caused by *Fusarium oxysporum* f.sp. *albedinis*. In Morocco, for example, more than 12 million date palms had been destroyed by 1994 (BENSLIMANE *et al.*, 1994). This reduction in the number of palms, impressive as it might be, does not reflect the only important effect of the damage. Bayoud has destroyed the world's most renowned varieties and particularly

those which have been given the best care and are the most productive (DJERBI, 1983). For this reason, combined with the destruction of date palms during wars of certain areas, the material for re-establishment is in short demand.

Another factor which is increasing in importance after the Bayoud disease as a threat to date palm plantations around the world, is the damage caused by the Red Palm Weevil (*Rhynchophorus ferruginens* Oliv.) (AL-JAGHOUB *et al.*, 2000). This pest bores into the heart of the date palm to nest, thus destroying the palm completely. Recently also, a similar pest named the African Palm Weevil (*Rhynchophorus phoenicis* F.), was found infesting two date palm plantations in the Republic of South Africa and Zimbabwe which were areas considered to be free of major date palm pests and diseases (ZAID *et al.*, 1999).

The genetics, morphogenesis, morphology and physiology of palms have been less systematically studied than that of other tree crops because their agricultural uses were not fully appreciated. Because of the dioecious nature of the palm and the time taken to reach adult flowering stage, breeding for disease resistance or for high yield is a long-term and an expensive project. Therefore, a serious interest in tissue culture as a means to mass propagate high yielding or disease resistant clones in large numbers for plantation cultivation has increased in importance over the past decade.

## **1.2 Economic importance of date palm**

The world production of dates has increased from about 1.8 million tonnes in 1961 to 2.8 million in 1985 and 4.8 million in 1996. In 1995, the top five producing countries were Egypt, Iran, Iraq, Saudi Arabia and Algeria (Fig. 2) accounting for 69 % of total production. During 1994, about 290,000 tonnes of dates were exported with a total value of about US \$ 250 million. When compared to the total figure of production, it shows that 93% of the dates produced are consumed within the producing countries. The major importing countries include France, India, The United Kingdom, Algeria, The Netherlands and The United Arab Emirates.

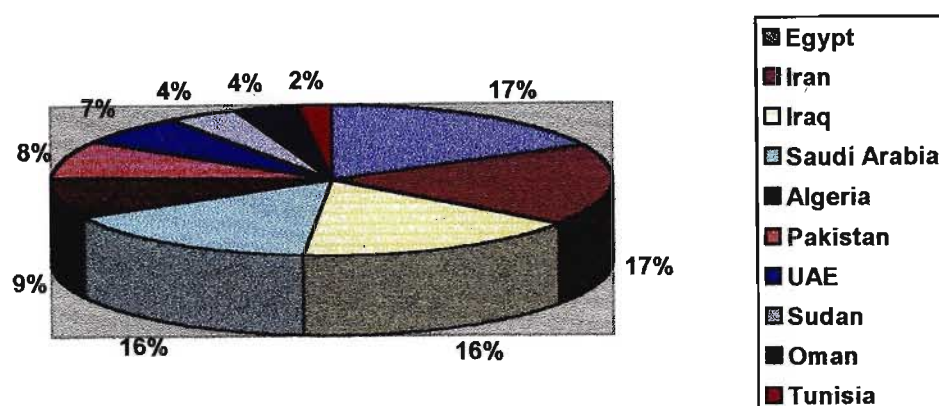


Fig. 2: Major date palm producing countries in the world and the percentage production.

Source: FAO Agrostat database (1995)

The date fruit (Fig. 1B), which is produced largely in the hot arid regions of southern Asia and north Africa, is marketed all over the world as a high value confectionery and fruit crop and remains an extremely important subsistence crop in most of the desert regions. Apart from its religious significance in the Middle East and North Africa, the date palm has become an important plantation crop due to the nutritious nature of the fruit. The dry flesh of the ripe date contains about 80% sugar, which in most varieties is made up mostly of glucose and fructose (NIXON and CARPENTER, 1978). Because the sugar source involved is not sucrose, the inverted glucose and fructose is easily digested and considered healthier. In first world countries, where organic foods and diet consciousness is increasing in importance, the date fruit is increasing in popularity.

The date fruit is consumed in various forms:

- Direct consumption while the fruit is in a fresh state (in the Khalaal and Rutab stages of maturity) in the summer of every year;
- Direct consumption of the completely ripe date during the rest of the year, and especially during the fasting month of Ramadan, Christmas and New Years when dates packed inside various containers are consumed;

-Indirect consumption of dates in confectionery, baking, and other products (Fig. 1C). They may be used for the production of vinegar, syrup, ethyl alcohol, protein yeasts etc.; and

-Consumption of inferior quality dates and pits for animal feeding.

A decline in productivity of the industry in the traditional growing areas over the last decade due to political, socio-economic and technical constraints, has created opportunities for the other under-exploited production areas of the world, including the southern African subcontinent.

The potential for date fruit growing in South Africa is great since there are not enough dates produced to even satisfy the local market (GOOSEN, 2000). Over 370 tons of dates are imported over the Ramadan period (a Muslim holy month where fresh dates are consumed after each daily fast), with the highest import price of US \$1.5/kg obtained in 1999. (HARIBHAI, pers comm.). Packaged dates obtained prices of US\$ 8.0/kg. With each palm producing up to 100 kg of fruit per season after five years, the investment is long term, but rewarding. Also, out of season fresh fruit can be exported to the Northern Hemisphere.

Although date palms are primarily cultivated for their fruits (Fig. 1C), many other parts of the palm can be used (Table 1). As many as 800 uses have been recorded in the Middle East (PURSEGLOVE, 1972).

The date palm also plays an important role in the ecology of various areas in the world environment. The palm provides protection to many associated crops such as fruit trees and annual crops from the harsh desert climates. Therefore, the date palm plantations not only results in income generation, foreign exchange and increased work and investment opportunities, but also assists in stopping desertification and gives life to deserts areas of the world.



Fig. 1A: Date palm (*Phoenix dactylifera* L.) plantation; B: Date fruit of a seedling palm;  
C: Date palm products on display

Table 1: Date palm products derived from various parts of the palm

<b>Date Palm part</b>	<b>Use</b>	<b>Reference</b>
Fruit	Jam Production Date Butter Dates-in-syrup Canned date pulp Date palm juice Confectionary (Baking) Sweetening and flavouring agent in ice cream Production of caramel colour from date juice Ethyl alcohol Vinegar Protein yeasts Animal feed Lipids Organic acids Single cell proteins Baby food	KHATCHADOURIAN <i>et al.</i> (1983) MUSTAFA <i>et al.</i> (1983) MIKKI <i>et al.</i> (1983) MUSTAFA <i>et al.</i> (1983)  HAMAD <i>et al.</i> (1983)  MIKKI <i>et al.</i> (1983)       BARREVELD (1993)
Seeds	Adulteration of coffee	
Palm leaves	Mulch Horticultural growing medium Thatch Partitioning in houses, roofing Religious festivals Baskets, sacks, woven products, Cords, brooms, bedding, upholstery, shade net	BARREVELD (1993)  BRANTON and BLAKE (1989)
Trunks	Building material Coarse flour	BRANTON and BLAKE (1989)
Midribs	Panel board products Brooms, fishing nets, Crates, Furniture, bird cages Rope, grape vine supports Fuel source, charcoal	BARREVELD (1993); FRERS (1983)
Spathes	Palm wine	BRANTON and BLAKE (1989)

### 1.3 Conventional propagation of date palm

Progress in the field of breeding, genetics, crop improvement and expansion of commercial plantings for the palm has been restricted by the habit and long-lived nature

of these monocotyledonous palms. For the date palm there are two methods for conventional propagation.

### **1.3.1 Seeds**

The date palm is dioecious and consequently about half of the progeny will be males and the other half females. Since the fruit are borne by the female palms (Fig. 3A) and only a few males are needed for pollination (Fig. 3B) (ratio of 1 male to 50 females) it is not economical to tender male palms. Also it takes several years to differentiate between males and females (approximately 4-5 years after planting) resulting in a waste of time, space and money.

However, in lieu of this problem, recent research using a cytological method based on chromomycin staining which demonstrates the occurrence of sexual chromosomes carrying distinctive nucleolar heterochromatin, offers for the first time an opportunity to identify male and female individuals (SILJAK-YAKOVLEV *et al.*, 1996). However, this technology is not available to all.

Propagation by seeds is useful for breeding but not ideal for commercial purposes since the material is not true to type. This is due to the heterozygous nature of the palm where there is much variation within the progeny. Desirable characteristics such as production potential, fruit quality and harvesting time of the parent plant may be lost as no two seedlings are alike.

### **1.3.2 Off-shoots**

The date palm is divided into two distinct developmental phases: vegetative, in which buds forming in the leaf axils develop into off-shoots (Fig. 3C) and flowering phase, in which the buds form inflorescences and off-shoot production ceases. Off-shoots are mainly produced during the early life cycle in a limited number depending on the variety of the palm.



Fig. 3A: Female inflorescences of date palm; B: Male inflorescences under infra red light for pollen collection; C: Off-shoots from vegetative buds of the date palm

Off-shoots, however, have the advantage of being true-to-type to the parent palm as the off-shoots are produced from the axillary buds of the mother plant and consequently the fruit produced will be of the same quality. Off-shoots also bear earlier than seedlings by 2-3 years. The number of off-shoots produced by individual date palms are highly variable. It is affected by cultivar and attempts to induce off-shoot production *in vivo* have been unsuccessful (MUNIER, 1973).

Disadvantages to off-shoot propagation are that whatever inert diseases or pests present on the mother plant, will be carried by the off-shoot and thus diseases such as the Bayoud, are spread to new plantations. Also, off-shoots do not have any roots and rooting *in vivo* can be problematic (EL-HAMADY *et al.*, 1992). Wrapping the off-shoot to induce rooting, separation and establishment of the off-shoot in the nursery does in some cases take many years (SCHROEDER, 1970). Off-shoots are often difficult and laborious to remove since they can weigh as much as 12 kg. Successful establishment in the soil is also very variable (30-80%) (VERAMENDI and NAVARRO, 1996). For importation of propagation material, the off-shoots are heavy and difficult to air freight and usually not free of soil. The maintenance during transportation, handling and inspection is also difficult and costly.

Apart from the limited multiplication of desired varieties, the cost of good off-shoot material is very high, being up to US\$ 400 per off-shoot, although US\$ 100 is more usual (BRANTON and BLAKE, 1989). Compared to the price of a tissue cultured plant of US\$ 25-35, off-shoots appear to be more expensive to purchase.

#### **1.4 Micropropagation of date palm**

Rapid propagation of date palms as well as propagation from a mature specimen is impossible due to the limited number of off-shoots available. The need to clone date palms is important since it provides a means of mass producing superior plants when material is in short supply. Tissue culture of date palms provides a method for such cloning.

True-to-type female and male plants are propagated from a selected mother plant with superior qualities. Plants are grown under sterile conditions and should be delivered free of disease. Plants are also uniform. Other advantages to micro-propagation of date palms are:

- the production of date palms all year round;
- exchange of disease and pest-free material between date growing countries;
- establishment of disease and pest -free germplasm and cryogenic storage;
- large scale production;
- hybridization and creation of new varieties and cultivars through genetic manipulation;
- introduction of disease resistance through genetic manipulation using micropropagation as a technical step;
- clones can be propagated from elite cultivars already in existence, or from the F1 hybrids of previously select, and seed only originated palms, as well as from strains selected based on yield; and
- a large number of plants can be cultivated in a restricted and small area for convenience of observation and maintenance and transported by air at a lower cost than large off-shoots.

Since 1970, many papers have appeared on the tissue culture of date palm (SCHROEDER, 1970; REYNOLDS and MURASHIGE, 1979; TISSERAT, 1979 and ABOEL-NIL, 1986). Table 2 outlines the explants used and the outcome of each experiment over the past 30 years. To date, commercial application of tissue culture in the propagation of date palms compared to other crops such as ornamentals, oil palms and fruit trees still needs much improvement.

Table 2: Date palm (*Phoenix dactylifera* L.) tissue culture describing explants used and types of development observed (x marks the positive development of plantlets or establishment of a free living plant).

Author	Variety	Source of explant	Type of development	Dev. Of plantlet	Estab. free living plant
Schroeder, 1970	Deglet Noor, Desert Nugget, Medjool, Honey	Embryo, Seedling Young leaf	Callus, Roots		X
Reuveni <i>et al.</i> , 1972	Zahidi	Embryo, Seedling	Callus		
Smith, 1975	Not given	Root		X	
Ammar & Benbadis, 1977	Deglet Noor	Cotyledons	Callus	X	X
Brochard, 1978	Deglet Noor	Root	Callus, Adventitious shoots		
Eeuwens, 1978	Not given	Leaf petiole, Stem	Roots		
Rhiss <i>et al.</i> , 1979	Bou-feggous, Bou-Sthammi	Apical meristem	Axillary buds	X	X
Reuveni, 1979	Not given	Embryo	Callus, Embryoid		
Reynolds & Murashige, 1979	Not given	Embryo, Axillary buds, Shoot tip, Inflorescence	Callus, Embryoid		
Tisserat, 1979	Not given	Embryo, Stem, Axillary bud, Shoot tip Inflorescence Roots	Callus, Embryoid  Roots	X	X
Tisserat <i>et al.</i> , 1979	Seedlings, Halawy	Buds, Shoot tips, Meristem, Embryos, Flower buds	Callus, Embryoids	X	X
Tisserat & De Mason, 1980	Deglet Noor	Embryo Axillary buds	Callus, Embryoids	X X	
Sharma <i>et al.</i> , 1980	Not given	Young leaf Seedling Embryo Mesocarp	Callus, Roots Callus  Callus	X  X	
Tisserat, 1981	Seedling, not given	Lateral buds	Callus, Plantlets	X	X
Tisserat <i>et al.</i> , 1981	Not given	Axillary buds	Callus, Embryoid	X	
Tisserat, 1982	Medjool	Axillary buds	Callus, Axillary shoots, Embryoid	X	
Zaid & Tisserat, 1983	Deglet Noor	Root, Stem, Young leaf Axillary bud, Shoot tip	Callus Callus, Embryoid		
Mater, 1983	Barhee, Halawy	Embryo	Callus, Embryoid	X	X
Drira, 1983	Kenta	Axillary meristem/buds	Adventitious shoots	X	X
Beauchesne, 1983	Bou Feggous, Bouskri, Bou Stammi noir cultivars	Axillary meristem/buds	Adventitious shoots	X	X

Table 2 cont.: Date palm (*Phoenix dactylifera* L.) tissue culture describing explants used and types of development observed (x marks the positive development of plantlets or establishment of a free living plant).

Author	Variety	Source of explant	Type of development	Dev. Of plantlet	Estab. free living plant
El-Hanawey <i>et al.</i> , 1983	Not given	Axillary and apical meristem	Shoots, Embryoids		
Khalil <i>et al.</i> , 1983	Helaly	Embryo	Callus, Roots		
Khan <i>et al.</i> , 1983	Not given	Axillary and apical meristem	Callus		
Ammar & Benbadis, 1983	Deglet Noor	Embryo Embryo	Callus Callus, Roots, Adven. Shoots	X	X
Gabr & Tisserat, 1984	Deglet Nour	Embryo, Shoot tip, Leaf Callus	Protoplasts		
Tisserat, 1984	Not given	Apical meristem	Axillary shoots	X	x
Zaid & Tisserat, 1984	Deglet Noor Sayer	Embryo Embryo	Callus, Leaf, Floral dev.	X	
Sharma <i>et al.</i> , 1984	Khadravi	Axillary and apical meristem Stem, Young leaf	Callus, Embryoids	X	
Gabr & Tisserat, 1985	Thoory Khalassa, Zahidi	Shoot tip Shoot tip	Callus, Embryoid, Axill. Callus, Embryoid	X X	X x
Omar & Arif, 1985	Maktoom	Ovules, Carpels, Flowers	Callus		
Omar <i>et al.</i> , 1985	Various	Lateral buds, Shoot tip, Leaf primordia, Immature ovules	Callus, Plantlets	X	X
Tisserat & De Mason, 1985	Not given	Embryo, Cultured plantlets	Axillary shoots		
Drira & Benbadis, 1985	Allig	Inflorescence	Callus, Roots	X	
Mater, 1986	Barhee, Hallawi	Shoot tip	Callus, Embryoids	X	X
Ammar <i>et al.</i> , 1987	Deglet Noor	Embryo	Floral dev.		
Dass <i>et al.</i> , 1989	Muscat	Shoot tips	Embryos		
Omar & Novak, 1990	Not given	Ovule segments	Callus, Embryos		
Veramendi & Navarro, 1996	Not given	Shoot tips and buds	Callus, Embryos	X	X
Falcone & Marcheschi, 1998	Deglet Nour	Shoot apex, Leaves, Radicals, Embryos	Embryogenic clumps	X	X

Date palm plantlets can be produced through either asexual embryogenesis via the initiation and germination of somatic embryos from callus, or from organogenesis, by the rooting and division of shoot tips and buds.

### **1.4.1 Somatic embryogenesis**

Somatic embryogenesis involves the establishment of calloid (indirect somatic embryogenesis) or secondary embryogenic cultures (direct somatic embryogenesis). The latter cultures are established from primary embryoids and consist essentially of parenchymatous nodules, each with numerous meristemoids within the corpus of parenchymatous cells. The meristemoids give rise to shoots.

#### **1.4.1.1 Explant sources and their morphogenetic responses**

A range of explants including shoot tips, buds, leaves, stems and roots were used from bearing palms, off-shoots, seedlings and asexual plantlets on medium containing  $100 \text{ mg l}^{-1}$  2,4-Dichlorophenoxyacetic acid (2,4-D) and  $3 \text{ mg l}^{-1}$  6-(*Y,Y*)-Dimethylallylamino purine (iP) which showed different morphogenetic responses (ZAID and TISSERAT, 1983). Leaf calli produced roots only, while shoot tip and buds produced adventitious shoots. Root explants failed to respond. The best callus and embryogenic responses were obtained from seedling and plantlet parent sources, while explants from off-shoots and mature palms were more difficult (ZAID and TISSERAT, 1983). This corresponds with the findings of MATER (1983) that immature tissues tend to produce callus rather than mature tissues.

Investigations on the size of the explant was conducted by GABR and TISSERAT (1985) where sizes of the shoot tip were trimmed between 5 mm and 1 mm in length. A shoot tip size trimmed to 3 mm was satisfactory for the growth of the shoot tips, while the smaller explants did not grow well in culture probably due to excision injuries. Larger explants were more prone to contamination.

*a) Plantlets derived from shoot tips and lateral buds*

Mostly, the shoot tip or meristem is used as an explant source for somatic embryogenesis since this area holds a source of meristematic undifferentiated cells that are fast growing and more likely to form callus than differentiated tissues. Shoot tip explants consisting of the apical dome and two to four leaf primordia were recommended as the shoots derived from these explants were consistently uniform compared to the plantlets derived from the apical dome only, which gave rise to deformed and irregular twisted leaves (ZAID and TISSERAT, 1983).

RASHID *et al.*, (1988) described a method using terminal buds as explants on 5 mg l<sup>-1</sup> 2,4-D. Callus was initiated forming green, nodular masses which was subdivided on a maintenance medium containing no plant growth regulators, however, this induced mainly root production. A surprising result was obtained by KACKAR *et al.* (1989) where callus was initiated after a mere two weeks on a slightly modified M&S medium containing (mg l<sup>-1</sup>): NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O- 170, glycine- 2.0, 2,4-D - 100, BA- 3, and activated charcoal- 0.3%, sucrose- 3% and agar (BDH)- 0.8%. The cultures were incubated in the dark at 28 °C. Somatic embryos were obtained three weeks later on the above medium devoid of plant growth regulators. KACKAR *et al.* (1989) attributed the early initiation of callus due to the younger age of off-shoots taken (1-2 years) and the size of the explant (1 cm). Because thin slender plants formed on the medium devoid of plant growth regulators, the embryos were transferred to a third medium containing M&S salts and 2 mg l<sup>-1</sup> each of  $\alpha$ - naphthylacetic acid (NAA), naphthoxyacetic acid (NOA) and benzyladenine (BA).

*b) Plantlets derived from highly differentiated tissues*

Using the soft white tissue at the base of the petioles as an explant source, growth was strongly stimulated by high concentrations of auxins (10<sup>-6</sup> to 10<sup>-5</sup> M 2,4-D or NAA) and sucrose (0.2 M) and in the absence of NH<sub>2</sub>Cl<sub>2</sub> by organic sources of reduced nitrogen (EEUWENS, 1978).

SCHROEDER (1970) reported callus development from seedling date palm leaves. The callus gave rise to roots several months later. Similar results with no further morphogenetic responses were observed by numerous authors (REUVENI *et al.*, 1972; EEUWENS and BLAKE, 1977; ZAID, 1987).

#### 1.4.1.2 Nutrient media

Some work has been done in optimizing media composition and incubation conditions. DASS *et al.* (1989) experimented with various media for callus induction and found that embryogenic cultures were induced on nutrient media containing MURASHIGE and SKOOG (1962) (M&S) basal salts along with NAA, 2,4-D, BA and Polyvinylpyrrolidone (PVP). Media supplemented with  $\text{NaH}_2\text{PO}_4$  and Kinetin was used to give rise to nodular embryos.

Shoot tip cultures were cultured on BA and Kinetin with 2,4-D or NAA. Only explants subcultured on high auxin containing media (10 and 100  $\text{mg l}^{-1}$ ) produced callus (MATER, 1986). Mature nodules were readily germinated within two months in one subculture on a medium containing 0.1  $\text{mg l}^{-1}$  NAA. Using 0.1  $\text{mg l}^{-1}$  NAA, embryogenic nodules elongated and first produced roots before the shoots were formed (DASS *et al.*, 1989). However, shoot proliferation was notably inhibited by 3-indolebutyric acid (IBA) and 4-chlorophenoyacetic acid (p-CPA) (ZAID and TISSERAT, 1983).

OMAR *et al.* (1985) found that although callus was initiated at lower concentrations of NAA and 2,4-D, 100  $\text{mg l}^{-1}$  2,4-D and 100  $\text{mg l}^{-1}$  NAA respectively gave the best results, with iP at 3  $\text{mg l}^{-1}$ . While high auxin levels stimulated callus growth, low auxin levels aided in shoot differentiation. Cytokinin did not aid in shoot differentiation at any level. Only 5-10% of the shoot tip cultures produced axillary bud outgrowths (TISSERAT, 1984).

Responses to various plant growth regulators using seedling sections according to (AMMAR and BENBADIS, 1977) were as follows: with Knobs medium there was no growth, and necrosis was exhibited. Sheaths bloated and callus was formed on media containing M&S salts with NAA and Kinetin, while inflorescences were produced on M&S

salts with IAA and BA. Callus was produced with roots and buds on a medium containing M&S salts with coconut milk, NAA and Kinetin. FALCONE and MARCHESCHI (1998) obtained somatic embryogenesis from various tissues using NAA ( $10 \text{ mg l}^{-1}$ ) and 2,4-D ( $1 \text{ mg l}^{-1}$ ). The medium, however, contained a very high concentration of sucrose (30%) to induce embryogenesis.

Alternative auxins, dicamba (3,6-dichloro-2-methoxybenzoic acid) and picloram (4-amino-3,5,6-trichloropicolinic acid) have been used in tissue culture with no adverse effects on callus or on subsequent regenerated plants. Maximum callus fresh weight was obtained by the addition of  $200 \mu\text{m}$  picloram (OMAR and NOVAK, 1990). Other auxins such as IAA and NAA for the production of callus were notably less effective than 2,4-D in callus production from cultured tips (GABR and TISSERAT, 1985).

Isopentenyladenine (iP) was the best cytokinin for regeneration from callus (ABOEL-NIL, 1986a). With the removal of callus from growth substances in the medium, normal plantlets were formed.

A further study was made on the effects of amino acid nitrogen on the growth of date palm callus. It was found that the growth of callus was stimulated in the following order: glutamine, asparagine, arginine, serine, glycine and alanine (ABOEL-NIL, 1986c). Basal inorganic media formulations such as Murashige & Skoog, Schenck & Hildebrandt (SH), Heller, White and Ericksson (ER) and Gamborg (B5) were tested for callus induction and plantlet regeneration by organogenesis and embryogenesis (ABOEL-NIL, 1986a). Callus induction was best on SH and B5 media while embryogenesis occurred on ER and SH media.

Activated charcoal added to the medium also plays a role in the growth and development of date palm cultures. The optimum concentration of activated charcoal used for the best vegetative growth of shoot tips and lateral buds, and differentiation of leaf primordia was between  $1.5$  to  $2 \text{ g l}^{-1}$  (ABOEL-NIL, 1986a; SHAHEEN and SAID, 1986). It is important to note that different explant types and different cultivars respond differently to various media (JASIM, 1998).

### 1.4.1.3 Acclimatization

One major problem area in date palm micro-propagation is the high percentage loss during the acclimatization stage. Laboratories experience between 40-80 % losses. This is a great area of concern when considering the time taken *in vitro* (two years) and low multiplication rates.

The conditions prior to transferring the plants to the greenhouse plays an important role in overall *in vivo* survival. TISSERAT (1979) showed that low concentrations of glucose, sucrose and glucose-sucrose in the medium prior to transplanting had an adverse effect on plant survival in the greenhouse. Glucose or sucrose at 3% gave the best acclimatization results. Relative humidity, roots and size of plantlets (should be greater than 10 cm) also played a role in overall survival percentage.

### 1.4.2 Organogenesis

This method relies on adventitious and axillary bud proliferation, beginning with isolated shoot apices or the collar region of aseptically germinated embryos (PARANJOTHY, 1982). Shoots produced from bud cultures may be due to the proliferation of axillary buds or by adventitious bud development (DRIRA, 1983).

Initially, shoot tip cultures were established on M&S medium supplemented with 100 mg l<sup>-1</sup> myo-inositol, 50 mg l<sup>-1</sup> adenine sulfate, 1.5 g l<sup>-1</sup> activated charcoal, 2 mg l<sup>-1</sup> iP and 0.1 mg l<sup>-1</sup> NAA. Direct shoot proliferation was observed after two subcultures onto M&S medium supplemented with 4 mg l<sup>-1</sup> iP, 4 mg l<sup>-1</sup> BA and 0.5 mg l<sup>-1</sup> NAA. Subsequent subculture of the proliferated shoots onto M&S medium containing 1 mg l<sup>-1</sup> NAA led to root formation (BEKHEET and SAKER, 1998). Proliferation of adventitious buds and some axillary bud multiplication was also achieved with M&S based medium containing combinations of iP (0.1 mg l<sup>-1</sup>), indolybutyric acid (0.5 mg l<sup>-1</sup>), indoleacetic acid (0.5 mg l<sup>-1</sup>) and naphthoxyacetic acid (2 mg l<sup>-1</sup>) (BEAUSCHESNE, 1983). M&S medium was found to support the best regeneration by organogenesis (ABOEL-NIL, 1986a). Precocious flowering was observed after five months in culture when BA, IAA and glucose were added to the medium (AMMAR *et al.*, 1987).

Reversion of floral primordia involved leaf like structures arising from the interior of the flower as well as from the axils of the sepals (DRIRA and BENBADIS, 1985). When shoots were produced amongst the carpels, there seemed to be a proliferation comparable to the production of shoots at the base of adult plants. TISSERAT and DE MASON (1980) however, found that floral bud reproductive tissue usually turned brown and died.

Direct organogenesis occurred from young leaves on media supplemented with  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  and low ratios of 2,4-D: iP (CHABANE and BOUGEDOURA, 1998). Young inflorescences were cultured on Gamborg and Eveleigh (1968) and Greshoff and Doy (1972) mineral salt solutions containing  $0.5 \text{ mg l}^{-1}$  NAA,  $2 \text{ mg l}^{-1}$  BA and  $1 \text{ mg l}^{-1}$  iP.

Apical dominance in the apical shoot tip was inhibited to allow for fast mini-shoot multiplication in a process labelled micromultiplication in Barhi (MATER, 1998). A modification of peroxidase activities and expression of isoperoxidase was found to precede the morphological appearance of buds and could be considered as a marker of the budding process (BOOIJ *et al.*, 1993).

BOOIJ *et al.*, 1993 states that due to the lack of observations on organogenic processes and the factors of its regulation, as well as the different growing requirements of numerous cultivars (as each behaves differently *in vitro*), much research is still required to improve on the techniques employed. Organogenesis in date palm also has a low efficiency rate. This is due to the low number of explants that respond *in vitro*, the long time taken for the initiation phase, lower multiplication rates and the strong influence of the variety on production and the effect of various media (BEAUCHESNE, 1983).

#### **1.4.3 Protoplast isolation**

Protoplast isolation from shoot tips does not seem to be possible, however, protoplasts derived from embryogenic callus regenerated cell walls, divided and even produced embryogenic callus with several embryoid developmental structures after 30 days in culture. However, attempts to reculture the callus failed (GABR and TISSERAT, 1984).

#### **1.4.4 Liquid cultures**

The best conditions for embryo development were found to be liquid media rather than a solid media (SHAHEEN and SAID, 1986; VERAMENDI and NAVARRO, 1996). Embryos placed in a liquid medium for a period of two weeks without sucrose, followed by culture on 3% sucrose contributed to embryo maturation. This was thought to be mainly due to the sucrose starvation period (VERAMENDI and NAVARRO, 1996).

Shoot tips were proliferated through axillary bud outgrowths on a liquid medium devoid of charcoal containing  $0.1 \text{ mg l}^{-1}$  NAA and  $10 \text{ mg l}^{-1}$  BA. Rooting occurred on solid media devoid of BA (TISSERAT, 1984). Similarly, an axillary shoot outgrowth phenomenon (20-30%) occurred from plantlets cultured on liquid medium containing various concentrations of BA ( $0.01$ - $1 \text{ mg l}^{-1}$ ) and IAA ( $0.001$ - $0.01 \text{ mg l}^{-1}$ ) (TISSERAT, 1982).

### **1.5 Problem areas in tissue culture of date palm**

Although methods for the micropropagation of date palm have been published, problem areas within the process still exists. A few of the problem areas are outlined below.

#### **1.5.1 Initiation**

##### **1.5.1.1 Explant source**

Cultures have been established from most parts of the date palm including embryos, axillary buds, pieces of stem, apical shoot tips and young leaves, however, only inflorescences have been used from mature palms. Once the palm stops producing off-shoots there is a need to be able to use parts of the mature palm for establishment. In some cases, there are a few or a single individual to be propagated from when off-shoots are very limited, or when some plantings containing valuable germplasm no longer produces off-shoots. A need therefore exists to be able to use more differentiated tissues from mature date palms, opposed to using tissues from off-shoots only.

Also, there is little detailed information on the behaviour of different explant types or the optimum conditions to produce embryogenic callus e.g. explant type and size. Work done by VERAMENDI and NAVARRO (1997) showed that although most types of explants produced callus, not all callus was embryogenic. The type of callus formed is also important.

Another problem is that the percentage of explants that form callus is low. In some cases only 7-14% of explants form callus (DASS *et al.*, 1989). The remaining cultures usually turn brown and die.

An alternative to 2,4-D as an auxin used for callus induction is sought due to several problems associated with its use in tissue culture such as chlorophyll inhibition, inconsistent plant regeneration and lack of genetic stability (ABOEL-NIL, 1986b). Both NAA and indole acetonitrile (IAN) can be used to replace 2,4-D as both support callus growth and regeneration of plants (ABOEL-NIL, 1986b).

#### **1.5.1.2 Contamination**

Decontamination of the date palm tissue is normally difficult (BRANTON and BLAKE, 1989). This appears to be a common problem to most initiation protocols where approximately 30 % of cultures are lost (VERAMENDI and NAVARRO, 1997). The contamination is usually due to bacterial micro-organisms and is also found to appear on many types of explants. Some authors have reported using mercuric chloride (DRIRA, 1983; SHARMA *et al.*, 1984) to obtain clean tissue. Bleaching with sodium hypochlorite to sterilize tissue has also been done (TISSERAT, 1979).

SCHROEDER (1970) also found contamination of explants a serious problem and reported that explants soaked in alcohol, then flamed greatly enhanced surface sterilization. Seeds were found to be a source of clean material, however, seeds are not used in commercial laboratories due to their variability. It thus can be used for research only.

OPPENHEIMER and REUVENI (1972) also surface sterilized lateral buds with a dip in alcohol and flaming, then submerged the buds in 3% calcium hypochlorite and rinsed in sterile water. The flaming was later replaced with dipping the explant in calcium hypochlorite for half an hour. The addition of antibiotics such as Penicillin -G and Mycostatin was ineffective. Explant tissues such as ovules, carple tissue, parthenogenetic endosperm, fruit stalks, mantle meristems and roots all turned black within 24 hours and died.

Surface decontamination of shoot tips *in vitro* at 2,63% sodium hypochlorite (NaOCl) was most effective, with lower concentrations resulting to inversely proportionate contamination. NaOCl was applied for 15 minutes and the explant rinsed three times with sterile distilled water (ZAID and TISSERAT, 1983). However, contamination was still problematic. Alternative methods and decontaminating agents need to be tested for better contamination control since the process of initiation is laborious and explant sources are limited.

### 1.5.1.3 Browning

Date palm tissue cultures when initiated are commonly known to release discolouring substances into the medium (SHANEEN and SAID, 1986; ZAID, 1986). The browning of the tissue and adjacent medium is assumed to be due to the oxidation of polyphenols and the formation of quinones, which are reactive and toxic to the tissues. Physical characters and cultural conditions play a major role in the browning problem. High temperatures (MONACO *et al.*, 1977) and increased pH of media from 2.5 to 5.6 is known to increase browning (ARORA, 1969).

In palm cultures browning appears to be promoted by cytokinins and agar (MARTIN and RABECHAULT, 1978). Methods used to control browning include frequent reculturing of small explants and/or placing freshly exised tissue into antioxidant solutions such as ascorbic acid and citric acid (ZAID and TISSERAT, 1983b) or ascorbic acid and cysteine (SHARMA *et al.*, 1984). A novel technique using paraffin wax to seal off the cut ends of the explant has been used to control browning in *Dioscorea alata* L. (BHAT and CHANDEL, 1991). This technique, although effective on most explant types, would not

be effective on meristems since it has to be exposed to high temperatures for a short period.

Activated charcoal (TISSERAT, 1979) and PVP (RHISS *et al.*, 1979) also inhibits browning of explants and appears advantageous for callus initiation. TISSERAT (1981) included activated charcoal in his recommendations for date tissue culture. T-dimethylsulfoxide and dihydrozaphthalene were found to be ineffective against browning in date palm explants (TISSERAT, 1979). Browning of the explants therefore still remains a problem during the initiation of callus and plantlets.

#### **1.5.1.4 Auxin concentration**

High concentrations of auxin, and in particular, 2,4-D, has been used for the production of callus initiation. It is not clear whether this is necessary, since 2,4-D is known to induce off-types in certain plants (see somaclonal variation below). It therefore becomes important to search for an alternative auxin used at lower concentrations, for the production of callus or embryos.

TISSERAT *et al.* (1979) used low concentrations of NAA and 2,4 -D for the initiation of shoot tips and buds. At low concentrations ( $1 \text{ mg l}^{-1}$ ), however, the cultures produced shoots, turned green and enlarged considerably in size, thus dedifferentiation did not occur. Generally, the ratio of callus formation increases with increasing auxin concentration e.g. 2,4-D (TISSERAT *et al.*, 1979). Other auxins such as IAA and NAA for the production of callus were notably less effective than 2,4-D in callus production from cultured tips (GABR and TISSERAT, 1985).

#### **1.5.2 Somaclonal variation**

The assessment of genetic stability of *in vitro* derived clones is essential in the application of biotechnology for micropropagation of true-to-type clones, for *in vitro* germplasm conservation and for commercial planting of high quality materials.

In many reports mentioned above, the auxin 2,4-D was used for callus induction. 2,4-Dichlorophenoxyacetic acid is known to be the most potent auxin available in tissue culture and may impose undesirable features on the cultured callus such as loss of differentiation ability with prolonged subcultures, chromosomal aberrations, mutations and endoreduplication resulting in polyploidy and possibly sister chromatid exchange (OMAR and NOVAK, 1990).

With the induction of date palm callus by using high concentrations of 2,4-D, there is the risk that the plantlets regenerated from such a process by embryogenesis may not be true-to-type. In a commercial laboratory where thousands of plantlets are generated from a single explant, stability is critical. Should an off-type occur that affects the fruit in any form, it will only be noticed four years after purchase from the laboratory whereby the plant has subsequently incurred substantial development costs.

In oil palm, three different types of abnormal development have been observed, two of which concern the sex of the flowers (CORLEY *et al.*, 1986). Possible causes of variation have listed somaclonal variation in callus cultures. Plantlets derived from callus cultures *in vitro* have sometimes exhibited genetic variation different from the parental clone in other species (GAMBORG *et al.*, 1970; LIU and CHEN, 1976). Other variations refer to the production of abnormal plantlets such as the observation of albino plants (SHARMA *et al.*, 1984).

After *in vitro* plants have been produced it is important that there is follow up on the uniformity of such plants. A study conducted by FERRY *et al.* (1988), of tissue culture plants produced using a technique of axillary bud proliferation, observed for five years, showed that a rejuvenation phenomenon of the foliage occurred when compared to the foliage of off-shoots. Due to the concern of off-types in tissue cultured date palms, much research has been done recently to try and detect such variations. Fingerprinting (SAKER and MOURSY, 1998) and RAPD analysis (LETOUZE *et al.*, 1998) has been done to distinguish between varieties and to seek conformity of the plants. Selecting primers to show polymorphism and grouping associations among the different varieties was achieved on 43 varieties in Morocco (HASSAN *et al.*, 1998).

An RFLP analysis of five date palm elite cultivars (Barhee, Deglet Nour, Khalassa, Khadrawy and Medjool) has been performed on off-shoot leaves surrounding the shoot tips used in initiation in tissue culture. With the total DNA digested by EcoR1 and hybridized with cDNA probes randomly selected from a cDNA library constructed from highly organogenic calli of cv. Boustammi Noire, and with a heterologous 1.7 kb nuclear rDNA fragment, amplified during a polymerase chain reaction of jojoba genomic DNA, discrimination between the cultivars was easily made (CORNIQUEL and MERCIER, 1994).

Two minicircular plasmid-like DNA's were isolated and characterised from date palm mitochondria (BENSLIMANE *et al.*, 1994), while AL-SHAYJI *et al.* (1994) isolated and analyzed total genomic DNA from the date palm in order to assist in genetic improvement of the varieties.

Cytological studies of palm cell cultures are few and even these are briefly reported. It would seem useful, particularly in view of the commercial potential of the date palm to build up an understanding of their chromosomal cytology in relation to such factors as clonal origin, growth rates, capacity for differentiation and media components. The value of the cytological studies would however, be limited by failure to detect changes at the molecular level (PARANJOTHY, 1982).

Cytological studies of oil palm callus (JONES *et al.*, 1982) showed the anticipated deviations with some cells showing high ploidy levels and others with fractional DNA levels and aberrant metaphases. Of importance is that the cultures containing a proportion of aberrant cells still appeared to give rise to strictly diploid embryoids. Both seedlings and regenerant plants have been shown to contain a low percentage of polyploid cells in their roots (TAN, 1976), but embryoids appeared strictly diploid. It appears that a self regulating mechanism, ensuring development from diploid normal cells, may be operating in embryoid formation (BRACKPOOL *et al.*, 1986). Findings such as these have led laboratories to believe that embryos are less unstable compared with callus cultures and continuous embryo cultures have been used in some commercial companies.

Overall, it is important that commercial companies ensure a true-to-type product. Methods used in the tissue culture laboratory need to be assessed and type of variants determined. To ignore the possibility of somaclonal variation could result in massive financial losses and embarrassment.

## 1.6 Objectives

From a commercial point of view, there are a number of areas that need further study in order to recommend a culture medium or the choice of the best explant and media for callus initiation and regeneration. There is insufficient published material outlining the best method for propagation of date palm whether this is through somatic embryogenesis or organogenic methods. Also, since the period taken for the date palm to produce fruit and establish a production record is long, field trials based on the various methods are still in process. The demand for various varieties is on the increase and the commercial tissue culture laboratories are encouraged to produce more plants with lower production costs in reduced time.

This study therefore concentrated on *in vitro* methods to reduce the loss of micropropagated plantlets and improve on the micropropagation methods available. Problems such as the bacterial and fungal contamination, reactivity of explants and time taken during initiation were investigated. An objective was therefore to focus on the initiation process and to optimize the methodology.

True-to-typeness of date palms after several years in culture and the methods used in culture has been questioned. More recently, certain variations observed on some tissue culture plants poses a serious concern for the tissue culture industry and the purchaser of such plants. Most date palm farmers and organizations are ignorant of the problem areas associated with the purchase of tissue cultured plants and the information on their performance in southern Africa is lacking. A southern African survey of these "off-types" would be useful in assessing the current situation and in providing valuable information to such farmers and organizations (MCCUBBIN *et al.*, 2000).

Another objective of this study was to apply the three techniques available in the mass propagation of date palms i.e. direct and indirect somatic embryogenesis and organogenesis and to compare these methods. Although much work has been published on each technique individually, very little has been published on comparative analysis of the three methods, detailing the merits and drawbacks of each.

## CHAPTER 2

# A SOUTHERN AFRICAN SURVEY CONDUCTED FOR OFF-TYPES ON DATE PALMS PRODUCED USING SOMATIC EMBRYOGENESIS

### 2.1 Introduction

Date palms (*Phoenix dactylifera* L.) were initially introduced to southern Africa from seeds brought in by missionaries. The first commercial plantings were made using off-shoots purchased from Yuma, United States of America (NIEMOLLER, pers comm.). Tissue cultured plants for commercial plantings in southern Africa were introduced about a decade ago and most plantations are not yet in full production. However, because propagation by tissue culture allows for the transportation of a relatively disease free plant, true- to-typeness along with numerous other advantages, the purchasing of such plants has become popular.

Tissue cultured date palms are normally produced using two techniques: organogenesis and somatic embryogenesis (direct and indirect). Direct somatic embryogenesis of date palms involves the proliferation of embryo cultures while callus cultures are used in the indirect somatic embryogenesis technique. Organogenesis is the process whereby axillary or adventitious buds are proliferated. Since most of the plants planted in southern Africa have originated from plants produced via somatic embryogenesis, the author will concentrate on the latter.

The initiation phase of this technique is normally done by the introduction of high concentrations of the auxin 2,4-D with/without other plant growth regulators or combinations of other auxins and cytokinins into the plant growth medium of the explant. This induces callus and embryo development, which in turn allows for the mass production of plantlets. The callus consists of de-differentiated cells that grow in an unorganized mass. Should somaclonal variation occur at this stage, cells thought to be genetically identical turn out to be dissimilar, varying in height, colour, shape, disease resistance, and yield and maturation characteristics. This somaclonal variation has been reported in more than 30 species, including wheat, maize, tomato, banana, oil palm and potato. These spontaneous genetic changes occur in traits under simple genetic control,

such as grain colour in wheat to those under polygenic control such as plant height (MILLER, 1985). MEINKE (1995) describes fusca, leafy cotyledon, abnormal suspensor and embryonic pattern mutants.

Many mutants are defective in early stages of cell division and morphogenesis. Others fail to accumulate pigments and storage materials during embryonic maturation. Other mutants are likely to be defective in genes that play a more direct role in the regulation of plant growth and development. The challenge for the future is not only to identify genes that may regulate early embryogenesis directly, but even more importantly to determine how large numbers of genes interact to influence both morphogenesis and cellular differentiation throughout development (MEINKE, 1995).

When genetically normal tissues give rise to abnormal adventitious structures, it must be assumed that the conditions, media and growth regulators that have been provided are not optimal for morphogenesis. For example, the number of abnormal bud-like structures, such as pseudobuds and phylloids increase as the concentration of cytokinin above an optimum (GEORGE and SHERRINGTON, 1984).

Off-types often referred to, as "mutations" are a controversial issue among the tissue culture laboratories and the purchaser of such plants. When plants show abnormal plant growth behaviour it is important to determine whether such abnormality is as a result of environmental conditions or genetic aberration.

The fact that large numbers of plant somatic embryos can be generated in liquid media has been used to investigate early events in plant embryogenesis. A number of genes have been cloned that are expressed with varying degrees of specificity in zygotic and somatic embryos, and in some cases it has been established that the expression pattern is identical on somatic and zygotic embryos. Mutants disturbed in zygotic embryo development have been described that either die or has altered patterns of embryo development. Somatic cell mutants with altered or arrested embryo phenotypes have also been described (TERZI and LOSCHIAVO, 1990). In most cases the genes affected have not yet been identified, so although plant embryogenesis is morphologically well

described, our understanding of the molecular events that generate the plant embryo is still very limited (VAN ENGELEN and DE VRIES, 1992).

Abnormalities in plant growth with the use of 2,4-D on plants has been previously reported (LOUBSER, 1980). Unlike various other plant growth regulators such as IAA, which is broken down by the plant once absorbed, 2,4-D is sequestered by the plant and continually recycled. It has also been found that the 2,4-D effect is cumulative and because it remains in the tissues of the plants for long periods, the effects are long lasting.

With the induction of date palm callus by using high concentrations of auxin 2,4-D, the risk of the plantlets regenerated from such a process by embryogenesis may not be true-to-type exists. In a commercial set up where thousands of plantlets are generated from a single explant, stability is critical. Should an off-type occur that affects the fruit in any form, it will only be noticed four years after purchase from the laboratory whereby the purchaser has subsequently incurred substantial development costs. Therefore, to be able to assess the genetic stability of *in vitro* derived clones becomes essential in the application of biotechnology for micropropagation of true-to-type clones, for *in vitro* germplasm conservation and for commercial planting of high quality materials.

Due to this concern, some work has already been done to distinguish between varieties using RFLP, RAPD (CORNIQUEL and MERCIER, 1994; ALJIBOURI and OMAR, 1998; LETOUZE *et al.*, 1998) and representational difference analysis techniques (KUNERT *et al.*, 2000) on the date palm genome (BENSLIMANE *et al.*, 1994) (See Chapter 1).

A satisfactory process for the regeneration of date palm elite cultivars should result in individuals phenotypically and genetically identical to the explant from which they are derived. However, due to somaclonal variation generated during *in vitro* culture, it cannot be certified that the genetic organization of tissue culture derived material is identical to the mother plant or explant and that it is inherently homogeneous (EVANS, 1989).

The aim of the survey done in southern Africa was to report on the types of abnormalities and give possible reasons for such off-types as well as creating an all-important awareness for detection of abnormalities within an owner's plantation. The techniques used in the production of such plants will also be discussed.

## 2.2 Materials and Methods

A survey form was issued to over 30 date palm farmers in southern Africa. This included countries Zimbabwe, South Africa and Namibia. The questionnaire dealt with the origins of the date palms and any abnormalities that had been observed.

## 2.3 Results and Discussion

The survey conducted among farmers and companies revealed the following:

<b><u>Type of abnormality reported:</u></b>	<b><u>Percentage of purchased</u></b>
Variegation of leaves	0.005
Seedless fruit	0.1
Broader leaves and different spine structure	1.4
No pollination in Barhee variety, various degrees up to	100
Bending of stem	1.5
Compact growth, broad leaves	4

Date palms generally have a stable genome and off-types are rarely seen. However, with an increasing amount of tissue culture plants being planted it is necessary to understand which variations are as a result of the tissue culture process and which are not. From the above surveys, natural or environmental factors have to be taken into account.

The variegation of leaves was initially seen in the greenhouse. Among 100 000 plants produced; only 5 plants showed distinct variegation. Unlike general yellowing of the leaves caused by iron deficiencies, the leaves had a cream coloured stripe running parallel to the leaf margin. The variegation occurred on all leaves but in different forms

of stripes, some wider than others did. The plants were otherwise healthy and green. However, plants did not grow at the same rate as the normal plants. The environment was not different to the rest of the other plants produced (same water, media and nutrition given).

Variation as an off-type is common among other tissue culture produced plants such as bananas (ROBINSON, 1996), where a proportion of the plants are without chlorophyll. These plants are also sometimes referred to as partial albinos. The leaves of affected plants are found to contain plastids, which fail to develop into chloroplasts. Furthermore, the inability of plants to produce chlorophyll is now usually thought to have a genetic cause. Changes to both nuclear and chloroplast genes, and changes in ploidy (PARK and WALTON, 1989) may be involved. Possible other explanations may include virus/microbial contamination, *in vitro* media nutrient deficiency or the occurrence of chimeral albino sectors (GEORGE and SHERRINGTON, 1984).

The "seedless-fruit" palm (Fig. 4A) produced dates that were small in size, but very sweet to taste. The palm was also different in appearance from the other neighbouring Medjool plants. The farmer will be assessing the plant during the next season to see whether this seedless phenomenon is repeated.

The "seedless- fruit" date palm occurs singly in a plantation of 1000 identical Medjool date palms. The palm also does not produce any off-shoots, unlike most other date palms. It is different in appearance and the simplest explanation for this abnormality is that labels during delivery could have been mixed up and that this palm is of another variety. However, there is no knowledge of any "seedless-fruit" variety being introduced to South Africa at any time. As is well known, unfertilized fruit also have no mature seed. However, fertilization techniques applied were well managed and the orchard is generally well maintained.

Variation in the form of broader leaves (Fig. 4B) and different spine structure was seen among Medjool plants in the Northern Province of South Africa. The plants did produce fruit.

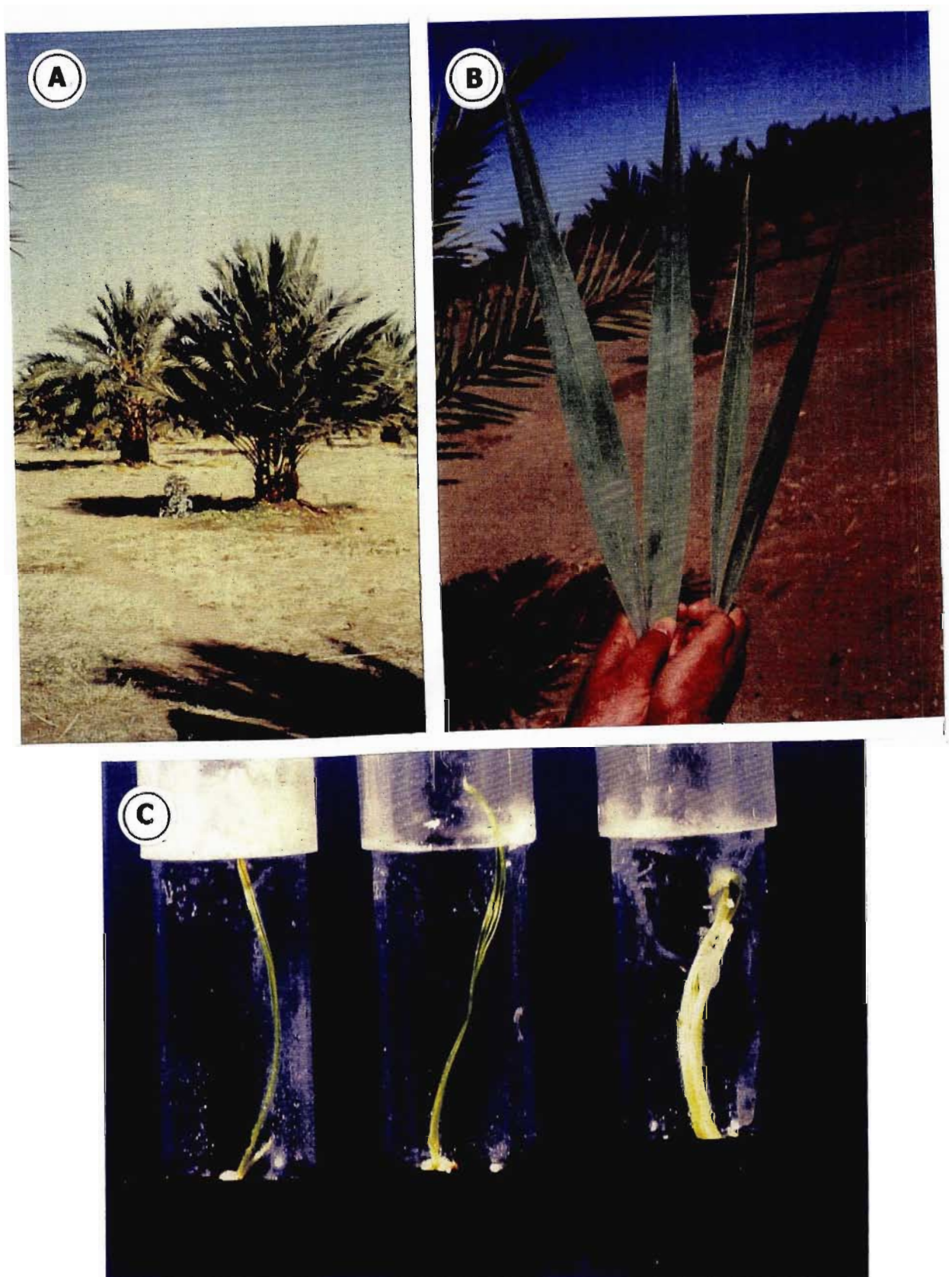


Fig.4A: Date palm that produces seedless fruit showing different growth pattern to the rest of the Medjool plantation; B: Broad (right) and narrow (left) leaves of date palm within the same plantation, showing growth variations; C: "Blind" or blocked meristem growth variation of date palm *in vitro* with flattened sheath and restricted leaf emergence

A report from Namibia also revealed similar abnormalities. The plants grew in a compact form (Fig. 5A) with broader leaves when compared to the surrounding Medjool plants with a normal growth habit (Fig. 5B). The plants showing such abnormalities were scattered among other plants in various plantations.

What is of interest is that the abnormalities have occurred on one variety- Medjool only, and that the plants were produced from three separate tissue culture laboratories all using somatic embryogenesis as a technique to propagate the palms. The plants were grown under different climatic conditions; different soils and management practices and have different sources of origin.

Pollination and subsequent fruit set problems have also been reported with Barhee tissue culture plants at various locations. Parthenocarpic fruits were formed and although the female flower was pollinated, the fruit appears unfertilized and have no seed. The fruit therefore do not mature and fall off. Instead of the normal three-carpelled inflorescence, five to six carpels were seen in some plants. The pollination problems occurred during the first and second productive year of the date palm. Pollination methods did not differ from the methods used for the other varieties within the same plantation which were successfully pollinated.

This abnormal fruiting occurrence derived from tissue culture plants propagated by somatic embryogenesis has also been reported to affect more than 100 000 date palm trees in Saudi Arabia (DJERBI, 2000). All pollinated bunches showed 80-100 % of parthenocarpic fruits.

A similar occurrence was reported in oil palm where three different types of abnormal development were reported, two of which concerned the sex of the flowers (CORLEY, *et al.*, 1986). It is also interesting to note that somaclonal variation normally occurs at the callus stage, but the abnormalities reported (e.g. mantled fruit) were from palms reproduced not from callus, but continuous culture of embryoids. This appears to be similar to the findings of the methods used in date palm as no callus cultures were used in the process of micropropagation of these plants.



Fig. 5A: Date palm var. Medjool (marked A) with broader leaves and a more compact growth habit than date palm marked B; B: Date palm var. Medjool showing normal leaf structure and growth

The use of 2,4-D in the medium may have increased the frequency of somaclonal variation, but the tissue culture laboratory that produced the date palms reported not using 2,4-D in the establishment medium. No 2,4-D was used in the culture of the two clones showing the abnormalities in oil palm as well (CORLEY, *et al.*, 1986). The use of 2,4-D or callus cultures can therefore be ruled out as a possible cause of the abnormalities.

In addition to the plant growth regulators and the process used for the production of plantlets being a factor, the temperature at which the *in vitro* cultures were grown may have influenced how rapidly the plants derived from them came into flower. Temperature manipulation during propagation may therefore also play a role (GEORGE and SHERRINGTON, 1984).

Prolific vegetative growth as a result of juvenile vigour may be another possible reason. Each leaf of the adult palm contains one axillary bud at the base. However, not all the buds develop and only when the palm attains sufficient size and vigour, usually around the fourth year of development, do vegetative axillary bud outgrowths (often called off-shoots or suckers) grow out of the parent trunk. Later still, axillary bud development is devoted almost exclusively to inflorescence production and off-shoot formation ceases (TISSERAT and DEMASON, 1985). Plantlets derived from somatic callus, however, develop precocious vegetative bud outgrowths after 2-4 months while still retaining their juvenile simple leaves, unlike the morphological events that occur in seedlings. These additional shoots develop from a common shoot axis (TISSERAT and DEMASON, 1985). Juvenile vigour, as described above, may explain a delay in pollination for one year; however, pollination should not be affected in subsequent years as were reported here.

There are a number of other possible explanations for the different growth pattern. These include, delayed flowering time related to establishment and size when compared to off-shoots, and high NPK inputs which may upset the balance between reproductive and vegetative growth. Drought, cold weather and insufficient microelement application of copper, zinc and boron may also be contributing factors to pistil development and fruit set. Genetic variation may also be a possibility.

There have been reports of delayed flowering in tissue cultured plants, such as the tree *Salvadora persica* where seedlings flowered in 20 months but tissue cultured plants only came into flower after 30 months (MASCARENHAS *et al.*, 1988). Similar cases have been reported on *Kalanchoe*, *Gypsophila* and *Dianthus*. However, although flowering was also delayed in the orchard described, it was the fertilization problem that was of most concern.

A number of farmers reported palms with stems that tended to bend. The bending did not all occur in the same direction. This phenomenon was investigated further and such plants were found to have symptoms of black scorch disease caused by *Thievaliopsis paradoxa*. The pathogen enters through a wound and progresses rapidly toward the bud. The entire terminal bud and adjacent leaf bases may decay, presenting a dried, dull and blackened appearance (DJERBI, 1983). Although this was not immediately observed, it is thought that the bending may be as a result of terminal bud decay. It appears that the tissue culture plants are more susceptible to this phenomenon than those plants introduced from off-shoots in South Africa. Good sanitation practices and fungal treatments may assist in the control of this phenomenon.

Variations observed in my study on *in vitro* plants include:

- dwarfs (thick plants with short, expanded succulent leaves);
- disproportionate shoot:root ratio;
- sheath restriction of leaf emergence (a leaf chokes the shoot tip). Upon dissection, fibrous tissue near the leaf base expands around the shoot tip leaving no room for the next leaf to appear; and
- 'blind' meristems (Fig. 4C), where the shoot meristem appears to be blocked or restricted (this phenomenon occurs in all techniques used);
- flowering *in vitro*.

In summary, the possible causes of abnormal development among the tissue culture plants can be broken up into various categories:

### 1) Environmental factors

These include disease, nutrition and climate.

## 2) Physiological factors

Tissue culture plants grow at different rates to seedlings and off-shoots. The vegetative phase in tissue cultured plants is more pronounced due to juvenile vigour. This is thought to be due to the build up of endogenous plant growth regulators after an *in vitro* propagation. To reduce this effect, a better understanding is needed of cytokinin and auxin levels within the plant and also of the level of plant growth regulators required *in vitro* to induce the necessary response.

## 3) Human error

Mislabeled plants has often caused much confusion and must also be taken into consideration. Inconsistent management of pollination, watering, fertilization and disease control practices could also lead to variation in growth responses within a plantation.

## 4) Tissue culture

### a) *Type and concentration of plant growth regulators*

The type of plant growth regulator used, such as 2,4-D, and the concentration at which it is used may contribute to somaclonal variation.

### b) *Type of process employed*

Proliferation of shoots and embryos is generally considered to reduce the risk of variation when compared to undifferentiated callus.

### c) *Length of time in culture*

CORLEY *et al.* (1986) suggests that the time for which the tissues are in culture may be an important factor in the abnormalities observed in oil palm plantations derived from the same population of cultures. The report by DJERBI (2000) seems to support this. Plants imported in 1992 with parthenocarpic fruits ranged from 30-40%; the problem increased considerably in intensity on imported trees in 1994-1995 with parthenocarpic fruits reaching 80-100%. Prolonged culture on media containing plant growth regulators may disrupt the normal hormone metabolism of the cells in some way. This disruption could persist in plants regenerated from the cultures, leading to developmental

abnormalities. Such effects are known to occur in other plant species, such as maize with gibberellic acid treatments (ROOD *et al.*, 1980).

*d) Type of mother material/explant used*

The mother plant must have a proven production record and originate from an orchard where sound management practices are exercised. Plantations where many unregistered herbicides, pesticides and fungicides are used should be avoided.

Although the variations reported concentrated on tissue cultured plants produced using somatic embryogenesis, the author cautions against any preference to plants produced by organogenesis until a thorough field investigation has been conducted on plants produced using both techniques.

## **2.4 Conclusion**

This study conducted in southern Africa shows that growth variations do occur from tissue cultured plants produced by somatic embryogenesis (direct and indirect). The frequency, with the exception of the Barhee pollination problem, is low (less than 5%). The factors influencing the variation in growth ranges from environmental and disease factors to possible genetic variation. Genetic variation can not be proved unless molecular technology is used to substantiate the visual assessments.

Although there have been various reports of variation in tissue culture plants produced by somatic embryogenesis (DJERBI, 2000; MCCUBBIN *et al.*, 2000), in contrast, there have been no such reports on the plants produced by organogenesis. Date palm farmers and organizations are becoming increasingly aware of this fact. In southern Africa however, because the date palm plants produced by organogenesis are still young, it is difficult to assess for variation at this stage. However, it is recommended that such a study be conducted as soon as the palms have reached bearing status.

It is not necessarily the type of process used in tissue culture that has led to such abnormalities, but possibly the length of time the cultures have been maintained in

culture. More frequent initiations producing fewer volumes of plants per mother plant would reduce the risk of somaclonal variation.

To quote TISSERAT, 1981: "Mutations and sports produced through tissue culture plants should not be ignored". Variation affecting production results in massive financial loss and bearing in mind that the date palm is a long-term investment, it is to be taken seriously.

Although much work has been done to develop tissue culture techniques on the mass production of date palms, much research is needed to verify the clonal nature of callus, embryo and organ- initiated plantlets produced *in vitro*. Date palm plantlets should be grown to maturity and the fruit quality and vegetative characteristics compared with the parental clone to determine their clonal status. Although this has been done by some tissue culture laboratories, the length taken to establish cultures, produce the *in vitro* plantlets, establish the plants in the field and then evaluate the palms at maturity can take between 7-10 years. To maintain cultures in the laboratory for such a long period would be risky and not always possible e.g. could be lost due to contamination.

Also, it is very important that farmers and organizations purchasing tissue culture plants are made aware of what is possibly an off-type and what is not. It is far too easy to blame any abnormality, be it a nutrient deficiency or disease on a so-called "mutation", demanding to be compensated for the loss. Published data, such as this study will help to provide professional information on the subject. Lastly, it is important that not all variability is to be regarded as an embarrassing and frustrating artifact of tissue culture, but instead, to be seen as a new source of traits and information for plant improvement as well.

## CHAPTER 3

# THE EFFECTS OF DECONTAMINATING AGENTS, PLANT GROWTH REGULATORS, EXPLANT TYPE AND NURSE CULTURES ON THE INITIATION OF DATE PALM AND SUBSEQUENT PLANT DEVELOPMENT

### 3.1 Introduction

Various reports on date palm tissue culture protocol optimization have been published (ABOEL-NIL, 1986a), however, problem areas still exist (ZAID, 1984). The limited success and slow progress in date palm micropropagation technology may be attributed to a lack of basic information and published data. The lack of knowledge of the factors controlling somatic embryogenesis, the asynchrony of somatic development, and low true-to-type embryonic efficiency are responsible for its reduced commercial application, but have been the driving force behind the fundamental research performed in the last decades (PEDROSO and PAIS, 1995).

The initiation of callus, embryos or plantlet clusters, which can take up to a year, is a long process when compared to other economically important crops. Problem areas such as contamination and browning of tissues (SHANEEN and SAID, 1986; ZAID, 1986) furthermore impede the process. Considering the time taken to initiate the plants and the subsequent monthly subculturing thereafter, it is important to know the best techniques to use in order to reduce costs. Some factors influencing cost of production include: multiplication rates, contamination, time spent on sterile transfers, and protocol. The objective of this study was to determine whether alternative methods (the use of various sterilizing agents, nurse cultures and plant growth regulators) could be used to speed up the process.

In most embryogenic cultures the small embryogenic cells co-exist with the large non-embryogenic cells. An important question therefore, is whether the presence of embryogenic cells is reflected by specific components secreted into the medium. A glycoprotein has been described that is produced only by non-embryogenic cells, while another secreted protein, identified as a lipid transfer protein, is produced only by

embryogenic cells and somatic embryos. Conditioned medium also contains proteins that disappear upon initiation of embryogenesis (EGERTSDOTTER *et al.*, 1993).

Over the years, many observations have indicated that cultured cells condition their medium with components that influence cell proliferation and somatic embryo development. A population of carrot cells newly released from the original somatic tissue becomes embryogenic much faster in a cell-free conditioned medium of an established embryogenic cell line (DE VRIES *et al.*, 1988). Coculture of one type of regenerating *Brassica* protoplast with a second type increased the division rate of the second type. This effect could be mimicked by medium in which protoplasts had been precultured. In addition the widespread use of feeder layers to improve plant cell proliferation indicates that cultured plant cells do indeed influence each other by means of secreted, soluble components. Changes in cell wall composition of competent cells occur shortly after the induction of embryogenesis (PEDROSO and PAIS, 1995). Studies revealed that media conditioned by plant cell cultures contain a complex array of molecules, mainly derived from the cell wall, including polysaccharides, proteoglycans and polypeptides (VAN ENGELEN and DE VRIES, 1992). Amongst the latter were a large number of enzymes, which fell into two main classes: oxidoreductases and hydrolases. Classic examples of the first group were peroxidases, which were implicated in crosslinking of wall components but were also capable of degrading auxins *in vitro*. Examples of the second group were glycosidases and endoglycanases. Possible substrates of these enzymes were the carbohydrate polymers of the primary cell wall and the middle lamella. The concept of a "nurse culture" or "feeder culture" was therefore used in this study to determine its effect on embryogenesis in date palm.

Furthermore, with various reports on the use of 2,4-D with its possible mutagenic long term effects on cultures (LOUBSER, 1980; GEORGE and SHERRINGTON, 1984), alternative methods and plant growth regulators were assessed as a possible substitute for 2,4-D in initiating callus. TIBA (2,3,5- Tri-iodobenzoic acid) is known to act as an anti-auxin or to inhibit auxin transport or movement. TIBA acts directly at the auxin receptor site from which it can displace auxins. If anti-auxins act only as auxin transport inhibitors, intracellular auxin levels could be expected to increase when both auxin and anti-auxin are presented to tissues. TIBA has been found to stimulate IAA uptake into

cells in suspension culture, particularly when the pH of the medium is low (pH 4.5) (GEORGE and SHERRINGTON, 1984). The effect of anti-auxins on somatic embryogenesis or embryo growth have therefore been equivocal. TIBA is more effective than normal auxins in inducing callus formation and embryogenesis (MOGHADDAM *et al.*, 2000).

A common problem in date palm explants is obtaining sterile tissues. Contamination of explants is high (VERAMENDI and NAVARRO, 1997; MCCUBBIN *et al.*, 2000) and many cultures are lost. Alternative decontaminating agents and methods to those reported (TISSERAT, 1979; DRIRA, 1983) were used in this study.

As mentioned in Chapter 1, the size of shoot tip explants has a significant effect on further growth. The size of explants other than shoot tips are also to be studied to determine growth responses and ultimately their potential to form embryogenic tissues. These, and many other, factors contribute to the date palm initiation process. It is therefore necessary to investigate as many factors as possible to reduce contamination and production losses.

## **3.2 Materials and Methods**

### ***3.2.1 Decontamination agents***

Male and female date palm off-shoots were removed as is shown in Fig. 6A and collected from Messina, South Africa. The entire off-shoot was dipped in HTH<sup>®</sup> (calcium hypochlorite as active ingredient) and fungicide (Benomyl). After removal of leaves, the terminal buds found at the base of each axil, the shoot tip, and its surrounding primordial leaves were excised and placed into chilled ascorbic (100 mg l<sup>-1</sup>) and citric acid (150 mg l<sup>-1</sup>) until they were surface disinfected. Inflorescence buds were discarded. The shoot tips and their surrounding leaf bases shown in glass flasks in Fig. 6B, were cut to an approximate size of 70 mm x 50 mm and surface treated using three methods:

- 1) 15 minutes in a 2.6% sodium hypochlorite solution containing 3 drops of Tween 20;
- 2) 10 minutes of full strength Bronocide™ (a bromonitro propane with active ingredient dichlorobenzyl alcohol) with added surfactant; and
- 3) 5 minutes 80% ethanol.

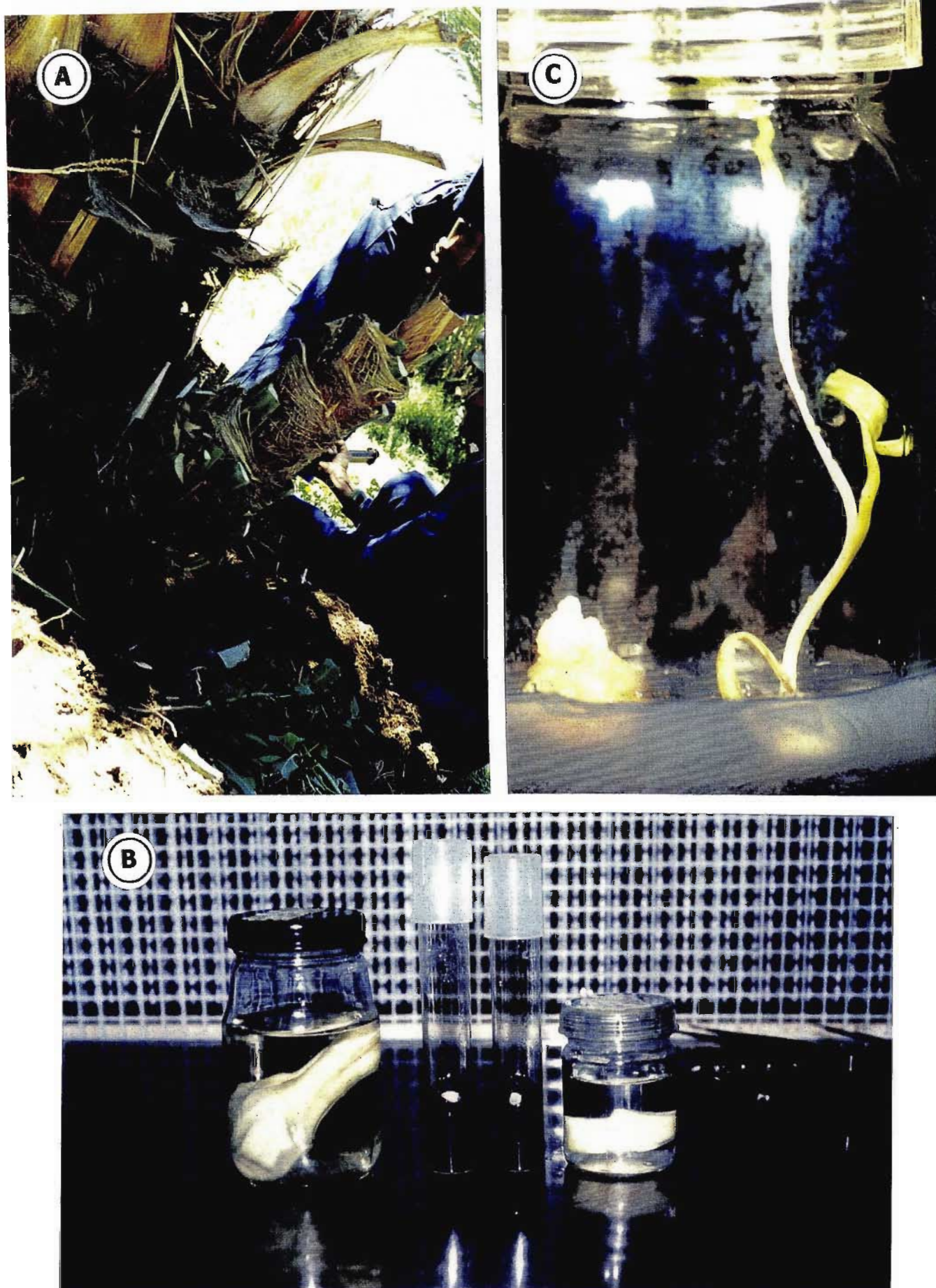


Fig. 6A: Date palm off-shoot being removed for initiation; B: Stages of date palm shoot tip and surrounding leaf bases initiation: first (left) and second (right) decontamination before inoculation on medium (middle); C: Two-leaf plantlet inoculated with date palm explant into initiation medium as a "nurse culture"

The explants were rinsed three times in sterile de-ionized water and then cut further removing the primordial leaves containing auxiliary buds surrounding the shoot tip. Half of the material was inoculated onto sterile media in glass culture vessels while the other half was decontaminated again for an additional 5 minutes in 1.3% sodium hypochlorite, followed by 3 minutes in 50% Bronocide™.

The shoot tip and some surrounding leaf bases were cut into quarters longitudinally and inoculated onto the sterile media. The media used contained Murashige and Skoog salts, Myo-inositol ( $0.1 \text{ g l}^{-1}$ ), thiamine ( $0.02 \text{ g l}^{-1}$ ), 0.3% activated charcoal, 3% sucrose,  $100 \text{ g l}^{-1}$  2,4-D,  $3 \text{ mg l}^{-1}$  iP and was solidified with agar-agar ( $8.5 \text{ g l}^{-1}$ ). The media was hot dispensed into glass vessels with screw lids (40 ml per jar) and autoclaved. The cultures were maintained in a temperature controlled growth room at  $22 \pm 1 \text{ }^\circ\text{C}$  in the dark. After one month, the amount and type of contamination was recorded. After 12 months the remaining cultures were counted again.

### ***3.2.2 Explant type***

The procedure was followed as above. The explants were allowed to grow for two transfers and then cut to the following sizes:

- 1) small (below  $1 \text{ cm}^3$ );
- 2) medium (between  $1\text{-}2 \text{ cm}^3$ ); and
- 3) large (larger than  $2 \text{ cm}^3$ ).

The explants were labeled according to their position from the shoot tip. As the tissues expanded, they were cut to size and inoculated onto sterile media.

### ***3.2.2 Plant growth regulators***

The procedure was followed as above, except that varieties of Medjool were collected from Venda, Pontdrift and Kakamas. Decontamination was performed with 2.6% sodium hypochlorite. The shoot tips and surrounding leaf primordia were inoculated onto the following media:

- 1) Murashige and Skoog salts, Myo-inositol ( $0.1 \text{ g l}^{-1}$ ), thiamine ( $0.02 \text{ g l}^{-1}$ ), 0.3% activated charcoal, 3% sucrose,  $100 \text{ g l}^{-1}$  2,4-D,  $3 \text{ mg l}^{-1}$  iP and solidified with agar-agar ( $8.5 \text{ g l}^{-1}$ ) (Table 4: Date palm somatic embryogenesis establishment medium).
- 2) Murashige and Skoog salts and vitamins, 0.3% activated charcoal, 3% sucrose, NAA ( $0.5 \text{ mg l}^{-1}$ ) and iP ( $2.5 \text{ mg l}^{-1}$ ) and agar ( $8.5 \text{ g l}^{-1}$ ).

In the third treatment the two above media were alternated every alternative subculture. Transfers onto fresh media were made every four weeks.

In a separate experiment, TIBA was used as an alternative plant growth regulator and compared to embryogenesis initiation medium (Table 4) and organogenesis establishment medium (Table 3) in its effect on inducing somatic embryogenesis or organogenesis of the date palm tissues. TIBA was dissolved in 1 M NaOH according to composition in Table 5. The medium was buffered to pH 5.8 prior to the addition of agar and autoclaved at  $121 \text{ }^\circ\text{C}$  for 25 minutes. Off-shoots from Letsitele, South Africa, of varieties Deglet Noor, Medjool and Khadrawy, were removed and initiated according to the procedure described above. Sodium hypochlorite was used as a decontaminating agent. Approximately 15 explants were obtained from each off-shoot. The explants consisted of the shoot tip, axillary buds and surrounding leaf bases. The cultures were incubated in the dark at  $25 \pm 2 \text{ }^\circ\text{C}$  and subcultured every four weeks onto fresh medium and placed randomly on a tray. Shoot growth and callus development were recorded.

### **3.2.4 "Nurse cultures"**

Date palm explants were initiated according to the procedure described above. Explants were inoculated into organogenesis (Table 3), somatic embryogenesis (Table 4) and TIBA medium (Table 5). The cultures were incubated in a growth room at  $25 \text{ }^\circ\text{C}$  in the dark for four weeks. Contaminated cultures were removed. Half the cultures were randomly chosen for re-inoculation onto fresh medium, while the other half were set aside for the incorporation of nurse cultures.

Table 3: Date palm organogenesis establishment medium

Component	Concentration (mg l <sup>-1</sup> )
KNO <sub>3</sub>	1900
NH <sub>4</sub> NO <sub>3</sub>	1650
CaCl <sub>2</sub> .6H <sub>2</sub> O	440
MgSO <sub>4</sub> .7H <sub>2</sub> O	370
NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	0.15
NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O	0.17
MnSO <sub>4</sub> .4H <sub>2</sub> O	22.3
ZnSO <sub>4</sub> .7H <sub>2</sub> O	8.6
H <sub>3</sub> BO <sub>3</sub>	0.62
KI	0.83
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.025
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.025
FeSO <sub>4</sub> .7H <sub>2</sub> O	0.025
Na <sub>2</sub> EDTA	37.35
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.25
KH <sub>2</sub> PO <sub>4</sub>	170
Skoogs Vitamins	
Thiamine HCl	10
Nicotinic acid	1
Pyridoxine	0.1
Biotin	0.001
Adenine	40
PVP	2000
Glutamine	200
Inositol	100
Ascorbic acid	75
Sucrose	30000
Agar-agar	8000
Plant growth regulators	
NAA	1
NOA	3
IAA	1
IP	0.1

Table 4: Date palm somatic embryogenesis establishment medium

<b>Component</b>	<b>Concentration (g l<sup>-1</sup>)</b>
M&S salts	
<b>Vitamins</b>	
Myo-inositol	0.1
Thiamine	0.02
Activated charcoal	3
Sucrose	30
Agar- agar	8
Plant growth regulators	
2,4- D	0.1
iP	0.003

Table 5: Date palm initiation medium containing TIBA

<b>Component</b>	<b>Concentration (g l<sup>-1</sup>)</b>
M&S salts	
M&S vitamins	
Activated charcoal	1
Sucrose	30
Plant growth regulator	
TIBA	0.025
Agar-agar	7

A mature date palm plantlet of the Barhee variety containing two green expanded leaves was inserted 2 cm away from the explant in the same culture vessel (Fig. 6C). The same plantlet was used for two subcultures, then replaced with a fresh plantlet. Cultures were observed for signs of callus initiation and somatic embryogenesis.

A plant growth regulator free medium (Table 6) was prepared. The medium was hot dispensed into 12 mm glass test tubes with polypropylene lids, autoclaved at 121°C for 25 minutes and allowed to cool on a sterile laminar flow bench. Before complete cooling took place, the test tubes were gently agitated to reduce the amount of activated charcoal precipitation. The media was allowed to solidify on a slant. Nodular callus was

transferred onto the medium and incubated in a growth room under low light intensity at  $25 \pm 2$  °C. Embryo development was observed and recorded.

Table 6: Date palm somatic embryogenesis shoot development medium

<b>Component</b>	<b>Concentration (g l<sup>-1</sup>)</b>
M&S salts	
Vitamins	
Myo-inositol	0.1
Thiamine	0.02
Activated charcoal	3
Sucrose	30
Agar-agar	8

### ***3.2.5 Plant development***

Somatic embryos regenerated from callus cultures were grown on media free of plant growth regulators for 2-3 subcultures until the leaves were fully extended and base thickened. The plantlets were then transferred to date palm somatic embryogenesis rooting medium I (Table 7) for root development. Once a primary root had formed, the plantlet was transferred to a liquid date palm somatic embryogenesis rooting medium II devoid of agar (Table 8) to encourage lateral root development. After eight weeks, rooted plantlets were transferred to the greenhouse where they were acclimatized in plastic pots. The disadvantages of each technique were recorded so as to ultimately decide on a technique to use that would benefit the propagator and to look at where there are areas of improvement.

Table 7: Date palm somatic embryogenesis rooting medium I

Component	Concentration (g l <sup>-1</sup> )
M&S salts	
Myo-inositol	0.1
Thiamine	0.004
Plant growth regulators	
NAA	0.001
Kinetin	0.001
Sucrose	30
Agar-agar	8.5

Table 8: Date palm somatic embryogenesis rooting medium II

Component	Concentration (g l <sup>-1</sup> )
M&S salts	
Myo-inositol	0.1
Thiamine	0.004
Plant growth regulators	
NAA	0.001
Kinetin	0.001
Sucrose	30

### 3.3 Results and Discussion

#### ***3.3.1 The effect of decontaminating agents on decontamination of date palm explants***

In this trial, sodium hypochlorite gave the best results. The explants that received two decontamination treatments had less contamination than those where the decontamination was not repeated. Only bacterial contamination was observed after a second decontamination. The fungal contamination had been successfully removed. Although Bronocide™ was effective in reducing bacterial contamination in other date

palm tissues, it was not effective with fragile tissue explants from the field. Most contamination observed after the second decontamination with Bronocide™ was due to white bacteria. The tissues treated with ethanol were all contaminated with bacterial and fungal micro-organisms. After one year in culture only the cultures that had been decontaminated twice in sodium hypochlorite survived. No improvement over standard sodium hypochlorite was found.

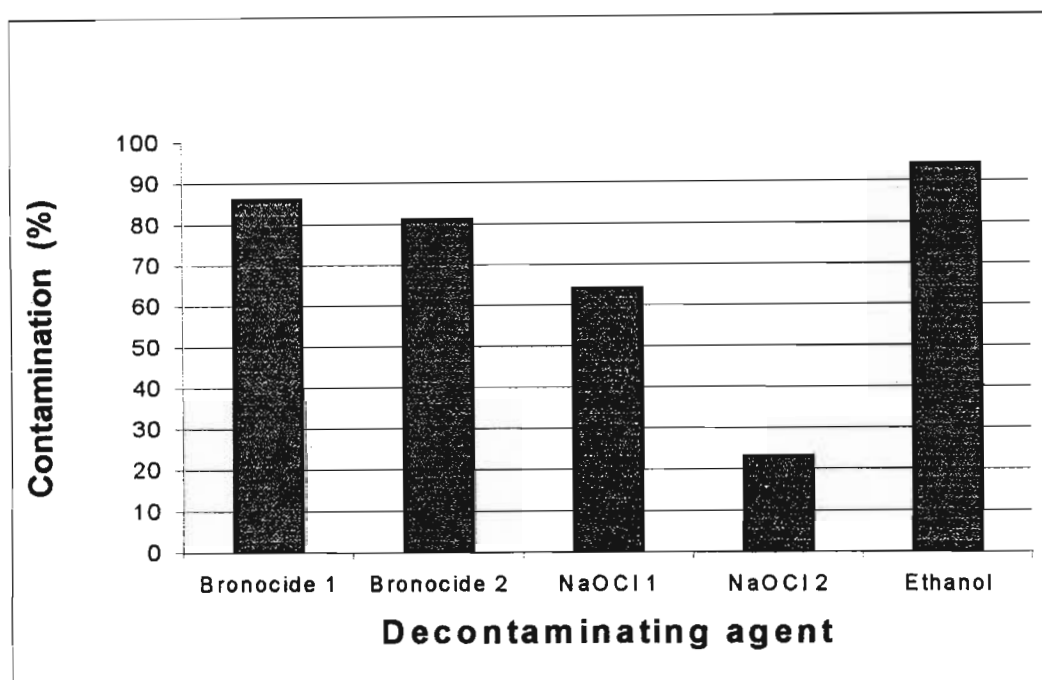


Fig. 7: The effect of various decontaminating agents on the contamination percentage of date palm explants. 1, 2 = one or two decontaminating treatments

### ***3.3.2 The effect of explant type on somatic embryogenesis***

Somatic embryogenesis and callogenesis occurred mainly from the shoot tip and a few surrounding primordial leaves (Fig. 8A). Experience with over 80 initiations showed that callogenesis occurred from pieces of material that were cut to 2-3 cm, so long as the meristematic areas were not removed in the cutting. Severe cutting to below 1 cm<sup>3</sup> resulted in a secretion of phenolics at the cut surfaces which caused tissue death (Fig. 8B). This is in contradiction to findings of GABR and TISSERAT (1985) where 3 mm

explants were used. Explants allowed to grow into larger pieces without cutting were more difficult to dedifferentiate, since such tissues tended to green up and differentiate into shoots (Fig. 8C).

### ***3.3.3 The effect of plant growth regulators on somatic embryogenesis and organogenesis***

No nodular callus or embryogenesis was observed for cultures inoculated with NAA+iP or those alternated with NAA+iP and 2,4-D+iP after 12 months in culture. Only cultures placed on 2,4-D+iP continuously, produced nodular callus and embryos. After one month, initial or primary callus formed (Fig. 9A). This callus was almost transparent and when transferred to medium free of plant growth regulators (embryogenesis multiplication medium), the callus turned brown and died. The initial/primary callus was not embryogenic. An embryogenic callus, nodular in appearance and creamy-white in colour formed (Fig. 9B) only after 5-10 months in culture. The callus viewed under microscope showed cells with large vacuoles (Fig. 9C). The cultures placed onto NAA+iP browned severely and tissue death occurred.

Explants placed on TIBA medium showed very little response besides a slight swelling of the tissues and the production of rhizoids (root-like outgrowths). These rhizoids (Fig. 9D) were white in colour and extended outwards above and below the medium. The rhizoids appeared to have a vascular cylinder in the centre when dissected. No callus was formed on the TIBA medium.

After 12 months, the cultures grown on organogenic medium produced embryogenic callus. This successfully proliferated on medium free of plant growth regulators and formed embryos. No shoot proliferation occurred after 12 months in culture. Most tissues died due to browning (Fig. 9E).

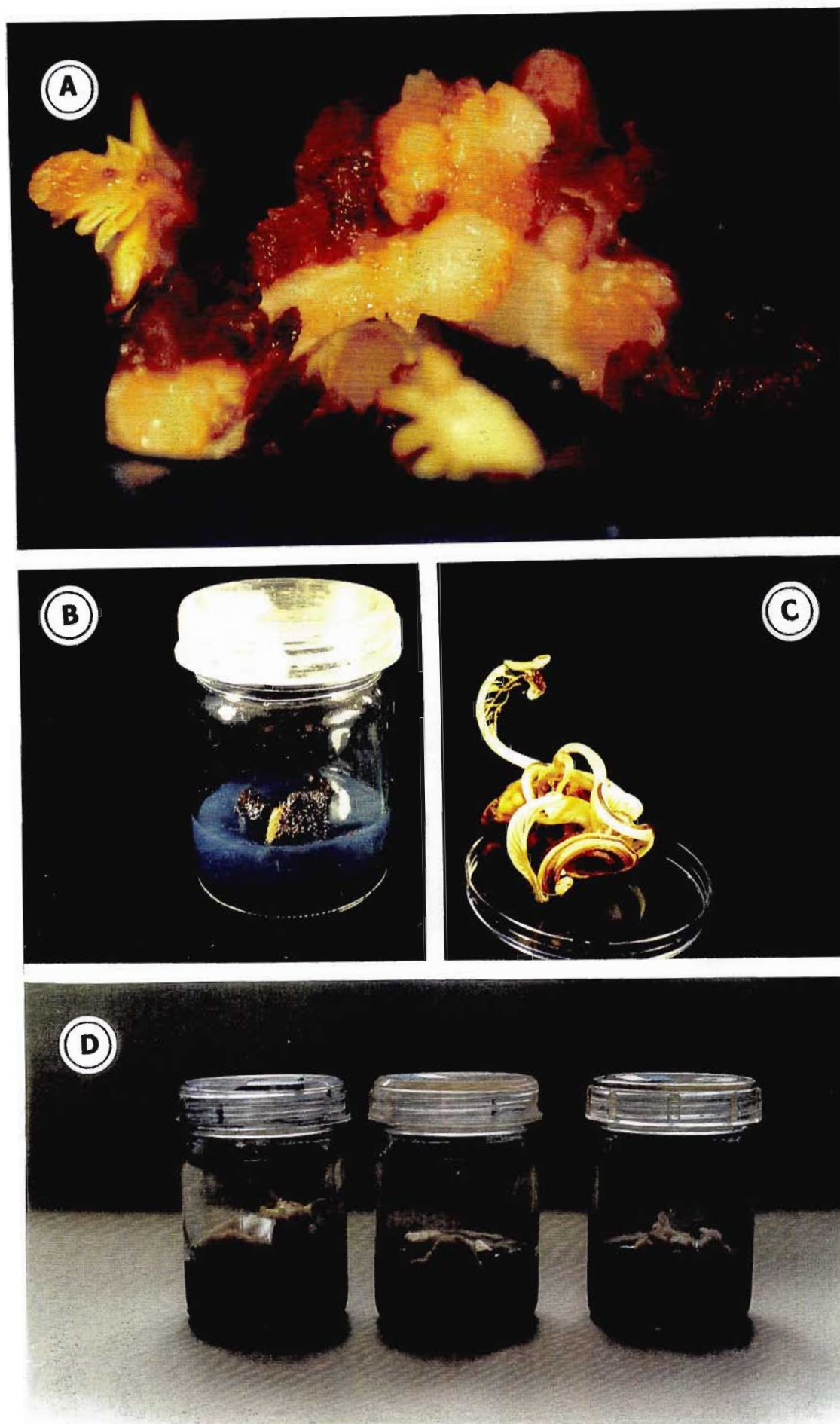


Fig. 8A: Callogenesis arising from shoot-tip meristematic tissue in date palm; B: Dead tissue due to blackening/browning; C: Date palm explant *in vitro* left uncut showing extensive growth and differentiation to leaves; D: Date palm explants showing size grading ( $> 1 \text{ cm}^3$  to  $< 1 \text{ cm}^3$ )

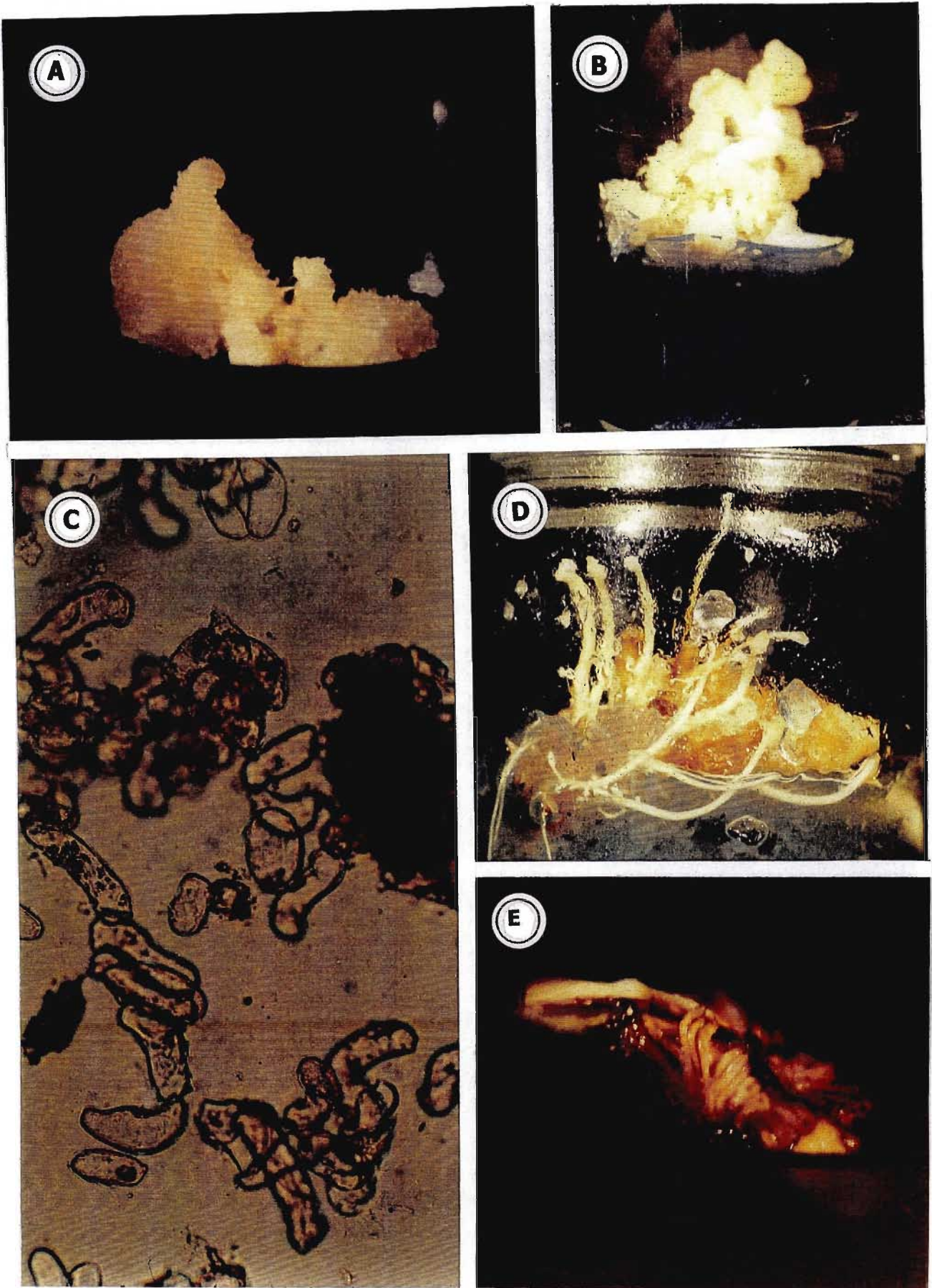


Fig. 9A: Initial primary date palm callus (non-embryogenic) formed one month after initiation; B: Friable, embryogenic date palm callus; C: Date palm embryogenic aggregates and cells with large vacuoles (Mag x 400); D: Date palm explant with root-like outgrowths grown on medium containing TIBA; E: Organogenic date palm explant showing browning of medium

### ***3.3.4 The effect of "nurse cultures" on somatic embryogenesis***

Results showed that the time taken to form nodular embryogenic callus with the "nurse cultures" was shortened by a number of months compared to the explants without the "nurse culture" (Fig. 10A). The time taken to initiate callus was five months for the "nurse cultures" while the other explants (excluding the shoot tip) took 12 months. The significant earlier induction of callus by the "nurse cultures" could be due to the release of secreted compounds into the medium that in some way stimulates somatic embryogenesis. Such effects on dedifferentiation have been previously recorded, but the exact mode of action is not known (VAN ENGELEN and DE VRIES, 1992). Earlier somatic embryogenesis could contribute to a reduction in costs and time in the tissue culture laboratory, thus leading to a quicker sale period.

### ***3.3.5 Plant development***

Continuous embryo cultures (Fig. 10B) produced embryo clusters and embryos which were separated from other "waste" material and subcultured again. Somatic embryos were also regenerated from callus cultures (Fig. 10C), while plantlets were formed on organogenic medium (Fig. 11A) as well. Shoot development was induced on the medium devoid of plant growth regulators and after 2-3 subcultures plantlets had formed 2-3 leaves. A primary root or roots were formed on date palm rooting medium (Fig. 11B) and lateral roots formed in liquid medium. The plants were successfully acclimatized in plastic pots (Fig. 11C) and formed a good root system (Fig. 11D).

The disadvantages and some advantages of the three micropropagation methods (direct and indirect somatic embryogenesis and organogenesis) used in date palm, are shown in Table 9.

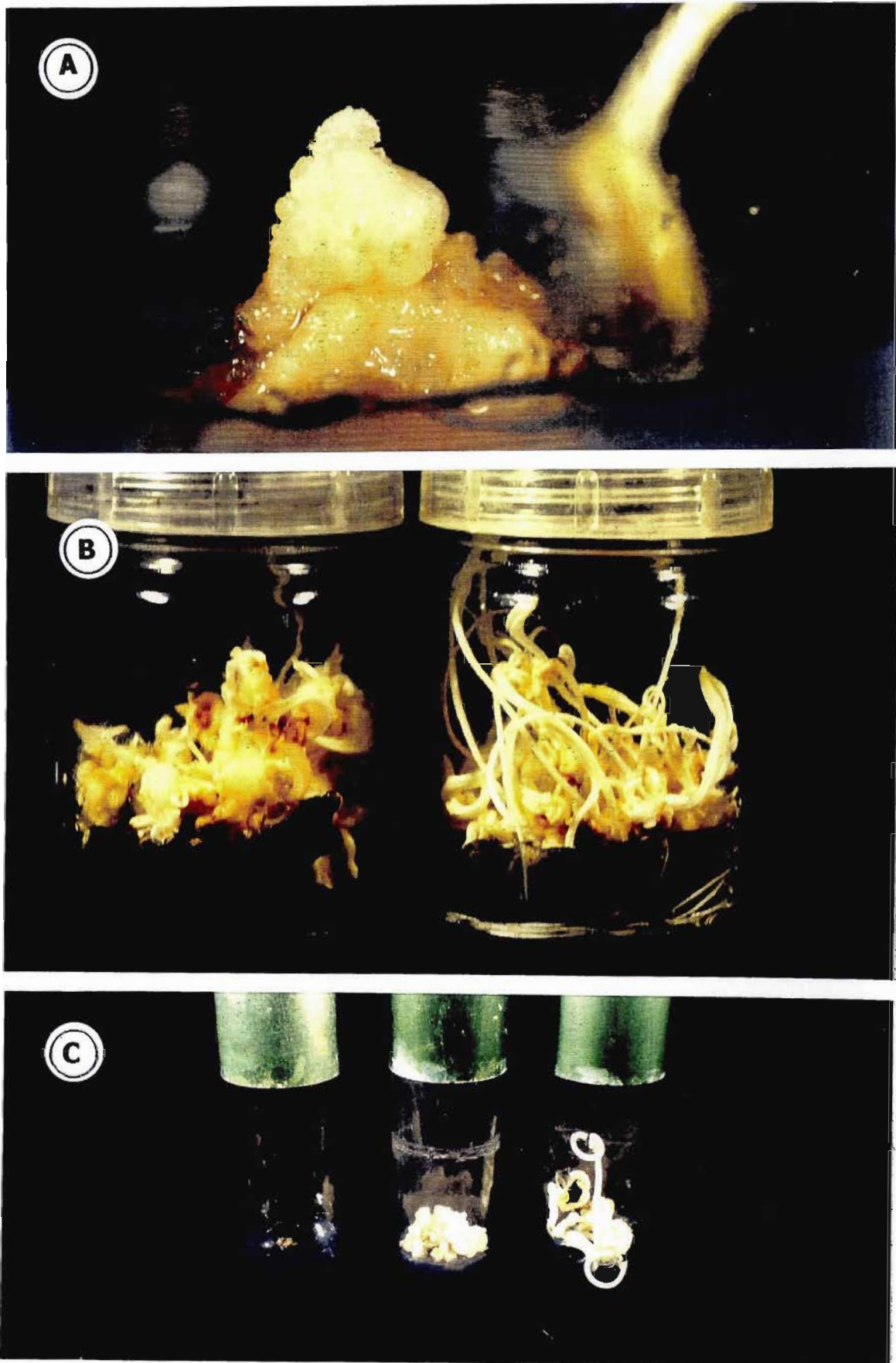


Fig. 10A: Callogenesis arising from explant of nurse culture in date palm; B: Continuous multiple embryo culture of date palm; C: Somatic embryos (Right) generated from proliferating callus cultures (left and middle)

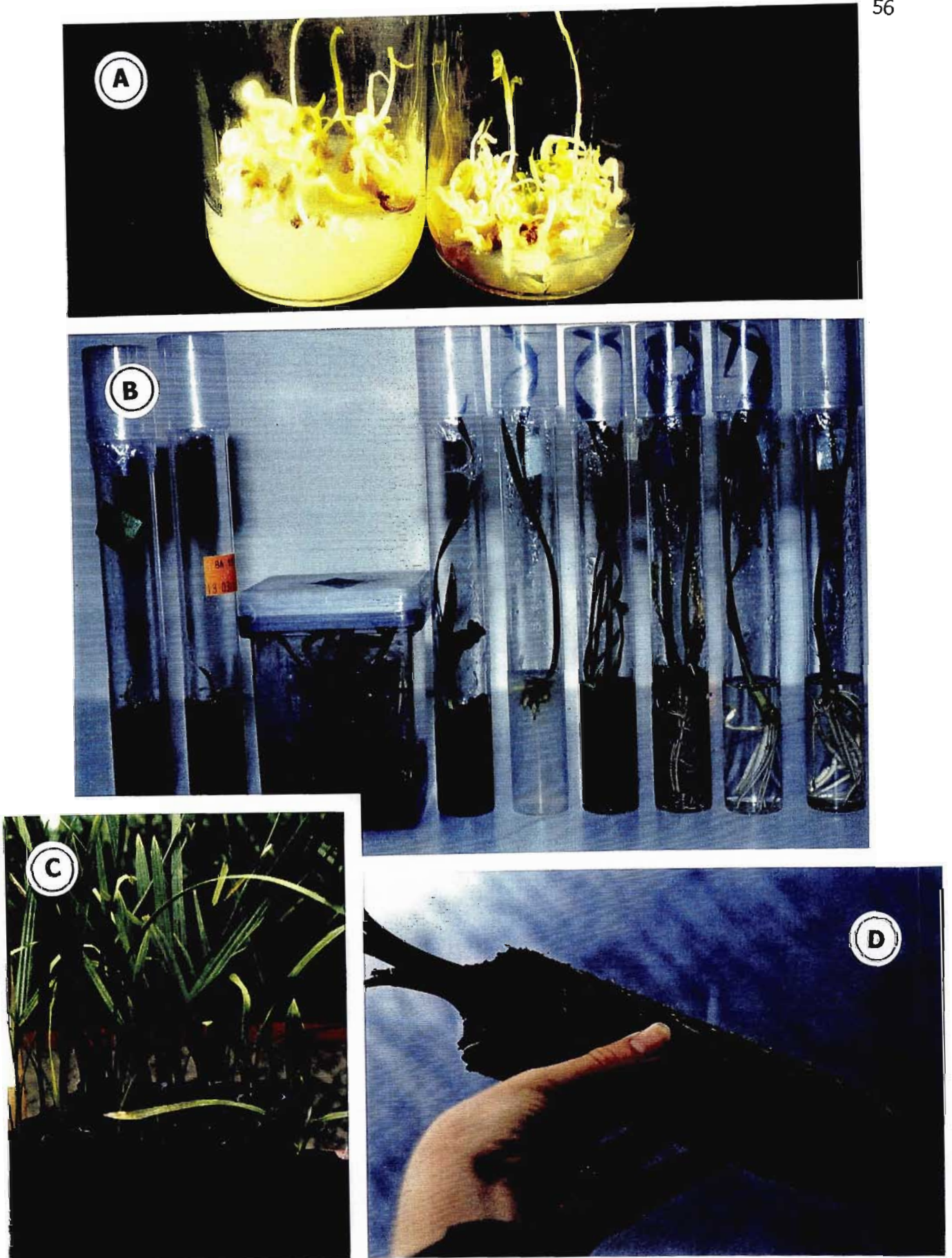


Fig. 11A: Multiplying organogenic date palm cultures; B: Date palm embryo development *in vitro*: shoot development (in activated charcoal medium) to rooting in agar medium (middle) to lateral root development in liquid medium; C: Acclimatized date palm plantlet in pots after 10 months in the greenhouse; D: Tissue cultured date palm plantlet with good root system

Table 9: Problem areas relating to the tissue culture of *Phoenix dactylifera* L. comparing the three techniques used commercially

<b>TYPE</b>	<b>Indirect somatic embryogenesis</b>	<b>Direct somatic Embryogenesis</b>	<b>Organogenesis</b>
<b>Initiation</b>	<ul style="list-style-type: none"> <li>*Destruction of off-shoot with the use of shoot tip meristem</li> <li>*Browning of explant leading to death of tissues (<math>\pm 30\%</math>)</li> <li>*Length of time taken to produce nodular callus and somatic embryos approximately 6-12 months</li> <li>* Use of 2,4-D in high concentrations increasing risk of somaclonal variation</li> <li>*Contamination losses</li> <li>* Varietal response differences</li> </ul>	<ul style="list-style-type: none"> <li>*Destruction of off-shoot with the use of shoot tip meristem</li> <li>*Browning of explant leading to death of tissues</li> <li>*Length of time taken to produce somatic embryos approximately 6-12 months</li> <li>*Use of auxins for dedifferentiation</li> <li>*Contamination losses</li> <li>* Varietal response differences</li> </ul>	<ul style="list-style-type: none"> <li>*Destruction of off-shoot with the use of shoot tip meristem</li> <li>*Browning of explant leading to death of tissues</li> <li>* Length of time for shoot proliferation is long (up to a year)</li> <li>*Contamination losses</li> <li>*Varietal response differences</li> <li>*Yield of the technique per off-shoot low</li> </ul>
<b>Multiplication</b>	<ul style="list-style-type: none"> <li>*Callus has limited life span and more sensitive to environmental changes</li> <li>*Risk of variation</li> <li>*Endophyte contamination</li> <li>*Vitrification of callus</li> <li>*Good multiplication rates (<math>\pm 7</math> per jar)</li> <li>*Unsynchronized embryo development</li> <li>*Loss of totipotency in some varieties</li> </ul>	<ul style="list-style-type: none"> <li>*Embryo proliferation is plant growth regulator and light sensitive</li> <li>*More stable than callus cultures and has an extended life span</li> <li>*Embryo selection is critical</li> <li>*Loss of totipotency in some varieties</li> <li>* Multiplication rates (<math>\pm 4</math> embryos per jar), variety dependent.</li> </ul>	<ul style="list-style-type: none"> <li>*Selection of plantlets is critical</li> <li>*Multiplication rates are low</li> <li>*Light sensitive</li> <li>* Loss of totipotency in some varieties</li> <li>* Precocious rooting sometimes occurs-decrease in regeneration capacity</li> </ul>
<b>Elongation</b>	Not necessary	Not necessary	Necessary Change in auxin concentration
<b>Rooting</b>	Low efficient rooting (2 stage rooting)	Rooting is generally not problematic (2 stage rooting)	Not all plants are rooted successfully
<b>Acclimatization</b>	Hardening-off percentage is generally 80% successful (location dependent)	Hardening-off percentage is generally 60% successful (location dependent)	Hardening-off percentage is generally high (90-98% success)(location dependent)

### 3.4 Conclusion

Various methods were used to optimize the initiation of date palm. A bromonitro propane product, Bronocide™ and ethanol was not effective in the decontamination of date palm compared with 2.6% sodium hypochlorite solution. A double disinfection of the explant with sodium hypochlorite was more effective in controlling contamination than a single disinfection. It was necessary, however to reduce the second disinfection treatment to half the concentration of the first disinfection.

The size of the explant played an important role in somatic embryogenesis and overall growth of the tissues during initiation. Explants cut smaller than 1cm<sup>3</sup> died due to browning, while the larger pieces (larger than 2 cm<sup>3</sup>) expanded and differentiated into organized tissues, thus becoming useless for somatic embryogenesis. The pieces cut to a size of 1-2 cm<sup>3</sup> were responsive. Somatic embryogenesis was found to occur from the shoot tip and some surrounding leaf bases more readily than other explant sources at initiation. Once the leaf bases have been removed from the shoot-tip at initiation, then the tissue is less likely to initiate callus. Outer leaf bases are also more prone to contamination and browning.

Alternate priming of 2,4-D plus iP followed by NAA plus iP on a monthly basis had no effect on somatic embryogenesis. Only cultures placed on 2,4-D plus iP continuously, produced embryogenic callus and embryos after 12 months. Following repeated subculturing of the aggregated yellowish callus (friable callus) on media containing high auxin levels, white embryogenic nodular callus was initiated from the superficial layers of the aggregates. These tiny nodules are precursors of asexual embryos and originate from small single meristematic cells in the callus tissue.

TIBA did not stimulate somatic embryogenesis at the concentration used (25 mg l<sup>-1</sup>). It is possible, that the addition of the low concentration of activated charcoal also may have had a diluting effect on the auxin (PAN and VAN STADEN, 1998). However, activated charcoal assists in absorbing the phenolics in the medium (WEATHERHEAD *et al.*, 1978) and should preferably be retained. Also, since TIBA is an "anti-auxin", it may have had a better effect if it had been added in conjunction with another auxin such as NAA, thus allowing for a better intracellular hormonal balance.

The addition of a "nurse culture" to an explant appeared to be beneficial in initiating earlier somatic embryogenesis in date palm. There are many advantages to reducing the initiation cycle such as decreased risk of contamination with less subcultures, decreased possibility of browning, increased production and faster output of a certain selection. It would be necessary, however, to use this technique on more than one clone to determine its effect on a range of varieties. Also, the type of "nurse culture" and medium:explant:subculture interval ratio needs to be carefully monitored. Depletion of the medium would lead to negative rather than positive effects.

Further research is still required to improve initiation techniques and cultural conditions of date palm. The use of an organogenic medium may reduce the risk of abnormalities later on, however, the process is slow and multiplication rates are low. With callus production on an organogenesis medium, the multiplication of embryos will be higher than the multiplication rates obtained for organogenic cultures, however, the multiplication of callus once again poses a risk of abnormalities later on. The solution may lie in the production of continuous embryogenic cultures from initials placed onto low levels of plant growth regulators such as those used in the organogenesis medium and where the amount of subcultures are limited. Each tissue culture laboratory will need to assess carefully which technique to use and by research, improve on the disadvantages of each.

## CHAPTER 4

### LITERATURE REVIEW OF PAPAYA MICROPROPAGATION

#### 4.1 Introduction

The Papaw (*Carica papaya* L.), also widely known as papaya belongs to the family Caricaceae and is believed to have originated in southern Mexico or in the tropical lowlands of the Caribbean Coast of South and Central America (STOREY, 1969). It is mainly suited to tropical climates although also grown in subtropical climates, as is the case in South Africa. Another member of the family, *Carica candamarcensis* and its hybrid *C. pentagona* (Babaco) is much more cold tolerant, however, the fruit is insipid and mostly eaten where the edible hybrid does not grow.

The papaya is an upright, soft-wooded, normally single-stemmed plant, rising as high as 10 m, but usually 3-4 m. The stem is hollow but segmented, having a diameter of some 20 -30 cm at maturity. The leaves occur singly on long petioles arising in a spiral fashion on the stem. The leaves are large, some 25-75 cm across, generally lobed, forming a cluster at the top of the stem.

The flowers can be borne in the axil of each leaf; however, this is controlled by temperature. Two general types of plants are recognized in the papaya. The first type is a dioecious plant where male and female flowers are on separate plants. The female flowers are small, creamy white and generally singular, borne close to the stem. The male flowers are borne on long thin branches and the flowers are numerous and smaller.

The second type of papaya is the hermaphrodite where male and female parts are carried together in one flower. The plants grown from seed of dioecious varieties will appear generally in the ratio of one female to one male. Pure hermaphrodite seed will give one female to two hermaphrodite plants. In each case there is no known way to predict the sex of the plant until the flowers appear. To complicate matters further, in cooler areas such as subtropical climates, hermaphrodite papayas change in flower type. The fruit change from pure hermaphrodite (which is an elongated symmetrical fruit) to

an intermediate form which gives rise to either distorted fruit or squat, ribbed fruit. The hermaphrodite types are generally less productive than the dioecious lines. Some male trees of the dioecious form also change flower types and set fruit that are edible and seed that perpetuate the variety of line more uniformly than seed from trees that are consistently female (CULL and LINDSAY, 1995).

## **4.2 Economic importance**

As postharvest and transport technologies improve, this highly perishable and easily injured fruit is growing in popularity on world markets. In 1993, it was estimated that almost 5.7 million metric tons of papaya fruit were harvested worldwide (FAO, 1993) which is worth approximately \$A 10-12 million annually in Australia (NFF, 1993). This amount is almost double that harvested in 1980.

The major producing countries and their respective production ('000 tons) are: Brazil-1750; India-1200; Nigeria-500; Indonesia-358; Mexico-340 and Zaire-200. In comparison, in 1992, South Africa produced 30 000 tons of fruit worth R 20 million per annum of which only a portion was exported (ALLAN, 1992).

The most popular cultivar at present in terms of economic value is the Sunrise Solo (Fig. 12A) with its origin in Hawaii which is small (450-550 g each) and easier to transport. The second most popular cultivar sold in South Africa is the Taiwanese Tainung no.1 (female and hermaphrodite)(Fig. 12B+C). This fruit is much larger and not economical to airfreight, however, has greater potential for the value adding market in prepackaged form (FISHER, pers comm, 2000). The papaya has great export potential. Major import markets for papaya are characterized by one dominant supplier. This is the case in the United States, which received the vast majority of papayas from Mexico, in Europe, which imports mainly from Brazil; and in Japan, where Hawaiian papayas are ubiquitous. In general markets for papaya have shown healthy, steady growth and many importers are optimistic about the future for this product (RAP market information bulletin, 1996).

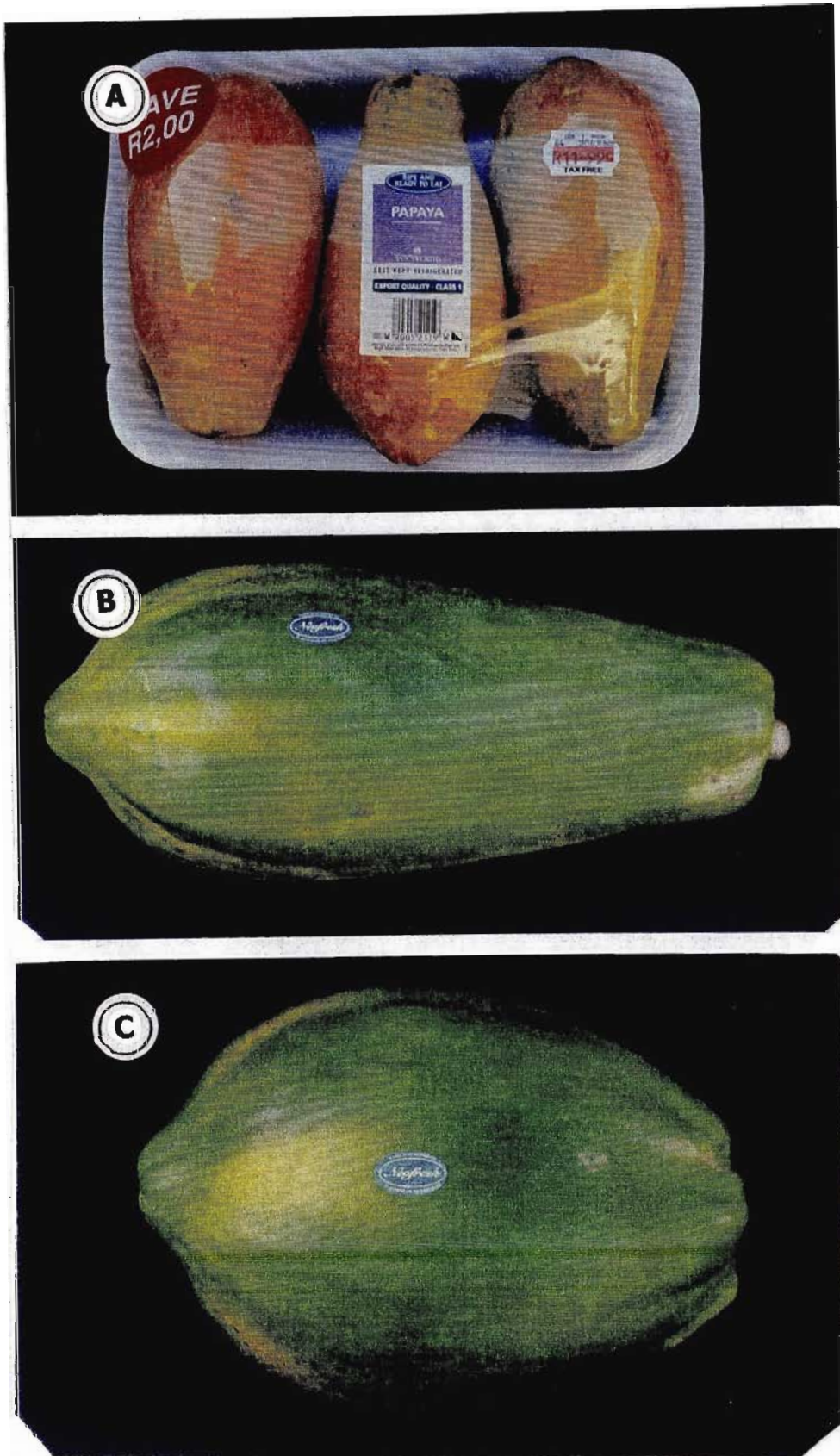


Fig.12A: *Carica papaya* L. var. Sunrise Solo fruit packaged for local market; B: Hermaphrodite fruit of *Carica papaya* L. var. Tainung no.1; C: Female fruit of *Carica papaya* L. var. Tainung no.1

South Africa currently exports most of its production to the United Kingdom and recently also to the Middle East. In the local market there has been good growth over the 8-9 years, where the papaya will beat banana sales during peak season and competes well with other fruit. An average of R18 is obtained for a 3.5 kg carton (D.I.P). South Africa exports approximately 3500-4000 tons of fruit per annum, of which 70% is of the Sunrise Solo variety, and 30 % exotic (FISHER, pers comm., 2000).

A survey conducted by Marks & Spencers (United Kingdom) revealed that only 30% of all fruit sold was exotic (melons, mangos, litchis and papaya). However, the growth rate on the 30% would be 70% towards the prepared food line and 30% in wholesale fruit. For prepackaged produce such as the incorporation of papaya into fruit salads etc., a growth potential of 45% per annum is estimated (FISHER, pers comm., 2000).

Besides being sold as fresh fruit, papayas are also used in pure fruit juices and juice blends. Dried papaya pieces and preserves that are less sticky, are also popular. Another by-product of the papaya is papain, an enzyme used for its digestive properties. The papaya is also highly nutritious, containing many macro- and micronutrients as well as Beta-carotene and vitamins B2 and C.

#### **4.3 Cultivar selection and breeding in South Africa**

Despite the papaya being a tropical fruit, it has become popular in the subtropics. Breeding more adapted genotypes can solve problems associated with this tropical fruit in subtropical conditions. The single most important characteristic to be addressed is tolerance to low temperatures which have a serious effect on the growth, fruit set and fruit quality. Paradoxically most of the fruit is harvested during the winter and spring from July to November in South Africa. Most fruit is set during the summer and start to ripen about six months later. The fruit qualities most severely affected by low temperatures are sugar content and internal fruit colour (LOUW, pers comm., 2000).

The fruit of the hermaphrodite trees are preferred and are of commercial importance. Hermaphrodite trees however, are sexually unstable. Under extreme temperature conditions, carpelloid flowers or male flowers are formed instead of the typical

hermaphrodite flowers. The carpelloid flowers give rise to malformed or cat-faced fruit, and the male flowers are female sterile, both abnormalities result in a loss of marketable fruit.

To eliminate the problems of unstable hermaphrodites, the obvious choice for the subtropics would be dioecious varieties. The dioecious varieties have therefore been the choice for breeding programmes in South Africa, Australia and the USA. Sunrise Solo, a small fruited cultivar which was bred in Hawaii has, however, become a world standard in fruit quality and a known tropical fruit in the import countries such as Europe and North America. The choice for an exportable papaya fruit suitable for breeding in South Africa would either have to be a stable hermaphrodite or a suitable dioecious variety. For the dioecious variety to become marketable, the fruit quality would have to be exceptional, as it is radically different in appearance to the known varieties.

In South Africa a formal breeding programme was started in 1931 by the late Dr. J.D.J. Hofmeyer. The programme was ended in 1942. In the early 1940's seed of "Hortus Gold", a cultivar developed in the breeding programme was distributed to interested farmers. The cultivar has sadly not been maintained and the name "Hortus Gold" became synonymous with the dioecious types of papaya rather than a cultivar. However, *Carica papaya* L. "Honey Gold" a vegetatively propagated female papaw and of dioecious origin, named at the University of Natal (South Africa) by Professor P. Allan (ALLAN, 1976), was developed from "Hortus Gold". This slower maturing, high yielding and good quality papaw is grown commercially in South Africa today (ALLAN, 1976), especially in KwaZulu- Natal.

In 1992 a survey was undertaken and collections were made from the main papaya producers. Most farmers had their own "brand" of papaya and in most cases less than 10% of the papaya bearing trees were producing a good crop. The breeding programme was reinitiated and promising trees were selected from commercial plantings. In 1994 the shipping industry showed interest in papaya as a possible crop for export as the peak production period of papaya being September to November, falls between the citrus and the deciduous fruit export seasons. At the same time a commercial nursery developed a technique by "microcuttings" to propagate papaya clones on a commercial

scale. The first cultivar to be propagated this way was named AF1. AF1 (a selection of Sunrise Solo) is a hermaphrodite cultivar with a slightly larger fruit than Sunrise Solo. The high yield of this cultivar and low percentage cat-faced fruit indicated that it would be possible to develop a hermaphrodite cultivar suitable for the subtropics (LOUW, pers comm., 1999).

Cloning techniques such as cuttings, micro-cuttings and tissue culture also paved the way for a new approach to the breeding programme. Instead of concentrating on the development of pure lines and synthetic hybrids, attention could also be given to individual plants as possible new cultivars.

Attributes that are important for selection include the following:

- The plant should set its first crop less than a meter from the ground on a sturdy stem.
- Fruit should not be distorted and too closely packed against the stem.
- Fruit should be uniform in size and 1-1.5 kg in weight.
- The shape should preferably be more elongated than round with straight sides with a flat base at the stalk end and a rounded top.
- The skin should be clean and free of blemishes and ripen evenly to a bright golden yellow and
- post-harvest qualities such as pink or red flesh with a firm rind is acceptable, while the fruit taste remains sweet and not bitter.

It is also important that the breeding programme includes resistance characteristics to major diseases in the papaya such as Freckle-spot (winter freckle), Powdery mildew (*Oidium caricae*), Black spot (*Asperisporium caricae*) and Ringspot virus. The breeding programmes so far has revealed many possible selections such as Baixinho (from Brazil) which retains its leaves in the winter, F1-2 selection, Red Lady, Tainung #5 and Tainung #1. Local breeding selection (Sel-42) with its very short internodes and late flowering also show promise (LOUW, pers comm., 1999).

To conclude, the available genetic variation in the breeding programme in South Africa is small. If the best characteristics of just three cultivars, Sunrise Solo, F1-2 and Baixinho

could be combined into a cultivar or cultivars, South Africa can develop a thriving papaya industry.

#### **4.4 Conventional methods of propagation**

##### **4.4.1 Seed**

Propagation is primarily by seed. However, careful selection of the tree from which the seed is taken must be practiced. With seed, it must be expected that most plants produced will not be as good as the parent.

Germination is usually best with fresh seed. Removal of the gelatin husk from the seed before planting is advised as it is thought to have germination inhibiting factors. When planted, the seed bed medium should not dry out until the seedlings emerge. To prevent damping-off the medium should then be allowed to dry-off. A fungicidal drench could be used. Seed should be well washed to remove all the pulp before storage. Seed should be stored dried in a sealed container kept in a refrigerator. Where seed is used for propagation material, 3-4 plants are planted in the same planting hole so that the males can be taken out later. Seed, however, is seldom used in commercial plantations, as it does not allow for uniformity and a good quality fruit for export.

##### **4.4.2 Cuttings**

To establish a plantation with uniform plants, cuttings may be used. These cuttings are taken from side shoots or shoots induced by decapitating the mother plant. The 300 mm long cuttings are cut from the plant and propagated in a coarse sand bed with bottom heat at 25-27 °C with intermittent mist and an enclosed cover. The surrounding temperature should be approximately 22 °C. The cuttings are prepared by removing all the leaves (not the petioles) except the growing point and one large leaf. Although cuttings provide a means to obtain clonal plants, when limited material is available, micropropagation provides a fast way of bulking up such material. Also, with cuttings, viruses such as the papaya ringspot virus, accompanies the propagation material.

#### **4.5 Micropropagation of papaya**

Cultivation of papaya is faced with problems due to its heterozygosity, dioecious nature and susceptibility to a large number of viral diseases. These features of the plant impose considerable limitations on improvement work and are some of the major reasons for the lack of true uniform varieties in this economically important tropical fruit crop (RAJEEVAN and PANDEY, 1986).

Micropropagation allows for true-to type uniform plants of a specific selection when the correct tissue culture methods are applied. Due to technical difficulties, few commercial plantings have been developed (DE FOSSARD, 1981). The tissue culture of papaya offers great potential for providing uniformity to support marketing initiatives aimed at increasing consumer use and confidence in the product. Also, the micropropagated papaya trees have a shorter juvenile stage than seedling trees. This has the advantage that less nodes have to be produced on the stem before flowering and fruiting commences. Fruits therefore begin to be produced earlier in the life cycle of the tree at only 30-40 cm from the ground (which makes harvesting easier). In seedlings, the main stem usually has to reach 1.5 -2m before flowering commences (DREW, 1988).

In commercial conditions, farmers have been advised to replant the plantation with new, young plants after three years of production. The plant becomes too tall to harvest the fruit easily otherwise. The plant can be rejuvenated by cutting the major stem 60-cm from the ground, allowing 2-3 suckers to grow, however, such plants do not produce any greater weight of fruit than the single stemmed plants and the fruit produced is generally smaller. The limbs often break off when carrying the crop. It is due to this replanting method that the production of large-scale, continuous quantities of tissue culture plants becomes commercially viable. The cost of production of a tissue cultured plant would have to be compared to the production loss if the replanting were not done or if inferior selections were used. For the tissue culture laboratory therefore, it is critical that the tissue culture process is not costly and that techniques are improved to keep such cost at a minimum.

*Carica papaya* L. has been micropropagated using various techniques and intermediary methods such as suspension cultures, the induction of somatic embryos, the proliferation of axillary shoots and subsequent microcuttings *in vitro*. These techniques are described below under the two subdivisions: somatic embryogenesis and organogenesis.

#### **4.5.1 Somatic embryogenesis**

Somatic embryogenesis employs the procedure of inducing dedifferentiation of the tissue leading to the formation of callus and embryos which are germinated and rooted to produce a hardened plant. Somatic embryogenesis offers a potential for high multiplication rates (each cell within the callus can potentially produce another plant) but suffers the disadvantage of possible genetic deviation from the mother material. Numerous authors have reported using this method of propagation on papaya for micropropagation and genetic manipulation.

LITZ and CONOVER (1981) used peduncles as an explant source and callus was regenerated on an NAA (1  $\mu\text{M}$ ) and BA (2  $\mu\text{M}$ ) supplemented solid medium (M&S medium with 3 g l<sup>-1</sup> sucrose and activated charcoal (AC)). Subsequent subcultures on medium containing no plant growth regulators and charcoal stimulated embryo and plantlet development. A clear conclusion from this study was that activated charcoal played a vital role in the induction of somatic embryos. The authors state that the phenolic inhibition of plant growth regulator action could have been absorbed by the AC and thereby stimulated somatic embryogenesis in *C. stipulata* cell cultures. NAA and BA were both essential for induction of somatic embryogenesis, but only when AC was also present.

FITCH (1993) obtained callus cultures of Sunrise and Sunset papaya cultivars using hypocotyl sections. Callus was obtained on medium containing half strength M&S salts with vitamin supplements, 60 g l<sup>-1</sup> sucrose and 45  $\mu\text{M}$  2,4-D. The embryos were grown in a liquid medium and then matured on a solid medium containing IBA. The embryos were placed onto a liquid vermiculite medium before hardening-off.

JORDAN and VELOZO (1997) showed that combinations of plant growth regulators NAA or IAA with BA, kinetin or zeatin triggered embryogenesis in *Carica pubescens* and *C. pentagona* (babaco). Axillary bud sprouting was inhibited by the presence of BA. 2,4- D only triggered cell suspension somatic embryogenesis. The cells were suspended in a Woody Plant Medium (WPM) for six days, then transferred to a growth regulator free medium under continuous agitation (50 rpm) for three months. Plantlets were recovered after transfer of mature embryos from cell suspensions into Magenta flasks. Initiation of embryos was achieved by the supplementation of 2,4- D (45  $\mu\text{M}$ ) alone or with Silver thiosulphate (STS) (50  $\mu\text{M}$ ). At the germination stage, the addition of STS (1  $\mu\text{M}$ ) increased the average number of papaya somatic embryos (ERNAWATI *et al.*, 1998).

CASTILLO *et al.* (1998) described a method of achieving 77.5 % successful encapsulated embryo germination. The use of a 2.5 % sodium alginate concentration in a half-strength M&S salt base significantly increased germination frequencies. Embryogenesis was also useful in achieving efficient transformation of papaya for papaya ringspot virus resistance. Papaya ringspot virus is a serious disease in many countries, which affects production. The coat protein gene of the ringspot virus was mediated by *Agrobacterium* following liquid phase wounding of embryogenic tissues with caborundum (CHENG *et al.*, 1996).

#### **4.5.2 Organogenesis**

Organogenesis adopts the procedure whereby shoot growth is induced. The shoots are multiplied and rooted. The rates of multiplication are not usually as high as that achieved by somatic embryogenesis, but has the advantage of ensuring that the resultant plants are normally identical to the plant from which they were derived (true clones). Much work has been done on the micropropagation of papaya using the organogenesis method as is reported below.

Sex type, seasonal responses and microbial infection (LITZ and CONOVER, 1981) affect establishment of explants. Using adult and mature field reproductive papaya plants with desirable traits as a source of explant material (axillary buds), appeared more difficult than using juvenile explants grown in the greenhouse (DREW, 1988; RAJEEVAN and PANDEY, 1986; LITZ and CONOVER, 1978). RAJEEVAN and PANDEY (1986) suggested

that the decapitation of field grown mature plants was impractical and had used lateral buds (5 mm) of the top young unexpanded leaves of mature trees as a source of explant material.

DREW and SMITH (1986) tried bud explants on LITZ and CONOVER (1978) media, but no shoot growth occurred. Riboflavin (Vitamin B12) interacted with IBA to initiate a rooting response where there was a degree of photo-oxidation (GORST and DE FOSSARD, 1980). Best shoot growth was achieved on modified DE FOSSARD *et al.* (1974) medium with  $1 \mu\text{mol l}^{-1}$  NAA and BA (DREW and SMITH, 1986). Riboflavin was found to reduce the amount of callus forming on the bases of the plants.

RAJEEVAN and PANDEY (1986) did studies on lateral bud culture of *Carica papaya* L. for clonal propagation. Lateral buds were taken from mature trees close to the apical bud. However, the lateral buds were extremely compact and had shortened internodes. In contrast to DREW and SMITH (1986), best growth occurred with Kinetin ( $50 \mu\text{M}$ ) and NAA ( $0.5 \mu\text{M}$ ). An average multiplication rate of ten was achieved with subculture intervals of 20 days. The major problem with the use of lateral bud culture for clonal propagation was the lack of shoot growth in association with the development of a normal root system. The extremely compact growth of the proliferating cultures and the failure of shoots to elongate even after rooting indicated the possibility of that the lateral buds were originally under strong control of apical dominance (RAJEEVAN and PANDEY, 1986). *In vitro* culture with cytokinins did not relieve the buds from the strong inhibition. Apical dominance could not be re-established even after 12-13 subcultures (LITZ and CONOVER, 1981).

DREW (1988) described a method of papaya propagation, which involved the removal of small axillary buds from branches of mature trees. These axillary buds were cultured initially on solid medium, and then on liquid medium on a roller drum in DREW and SMITH medium (1986) (DS medium) containing  $1 \mu\text{M}$  BAP +  $1 \mu\text{M}$  NAA. After two or three subcultures with alternate periods on hormone free basal medium, apically dominant shoots were produced. Axillary buds subsequently occurred on DS medium containing  $1 \mu\text{M}$  BAP +  $0.25 \mu\text{M}$  NAA. Roots were initiated on a reduced mineral medium containing  $10 \mu\text{M}$  IBA. This method was tedious and was improved (DREW, 1992). Here

the cuttings in the greenhouse were not grown out to six month-old plants but explants were taken from single node sections of a rooted cutting. Best multiplication rates were achieved when single node papaya sections were cultured on a modified DE FOSSARD medium containing 0.5  $\mu\text{M}$  of both BA and NAA. Shoots that developed from axillary buds were dissected and cultured for three days on a rooting medium containing 10  $\mu\text{M}$  IBA. The shoots were subsequently transferred to hormone-free DS medium (DREW, 1992).

Further improvements were made to the technique by the multiplication of nodal sections from apically dominant shoots, which had been grown in culture. Shoots developing from axillary buds were dissected and rooted as micro-cuttings. Optimizing exposure to auxin before transfer to hormone-free medium increased rooting percentages and quality of root systems (MANSHARDT and DREW, 1998).

## **4.6 Problem areas in the micropropagation of papaya**

### **4.6.1 Contamination**

Contamination is known to be one of the largest problems in obtaining sterile and continuous cultures *in vitro*. Contamination losses do not only have a financial impact, but also deplete resources and clone banks and affect production schedules.

A high level (80%) of bacterial contamination was found with bud explants from field grown papaya trees and most cultures were discarded (DREW, 1988). Initiations were thus done using very small bud explants by removing as much extra tissue as possible to avoid contamination (DREW and SMITH, 1986). However, with very little tissue left, growth was not good.

RAJEEVAN and PANDEY (1986) also reported bacterial contamination after subsequent subculturing. Loss in proliferation after 8-13 subcultures was also thought to be partially due to endophyte contamination.

It is therefore necessary to bring the contamination percentage during initiation to a minimum and to ensure that the cultures are well maintained in a controlled environment. The specific challenges for papaya are therefore to seek alternatives for decontamination and maintenance in culture to reduce endophytic bacterial contaminants. Endophytic contamination may not only cause total loss of the plant, but may also be present at low levels which retards growth. Harsh disinfection with concentrated chemicals can sufficiently disinfect, but may hamper further growth, while antibiotic treatments are expensive and not 100% effective. It is possible that by maintaining the plant in a medium that absorbs toxins and in turn maintains healthy growth of the plant, the incidence of contamination may be reduced.

#### **4.6.2 Multiplication**

Many problem areas occur at the multiplication stage. The first major problem reported was that of cytokinin toxicity after repeated subcultures. This caused a reduction in proliferation rates (DREW, 1988) severe abnormalities, and in some cases even death (LITZ, 1984). Shoot proliferation rates have generally been low in techniques applied possibly due to ethylene production (LAI, *et al.*, 2000).

BA toxicity in papaya has been characterized by the formation of pale leaves, prominent midribs and some misshapen leaves (DREW, 1992). As a result, DREW (1992) suggested that BA should be used at low concentrations and only to initiate growth of dormant buds. Subsequent growth was believed to be better achieved on hormone-free medium (DREW, 1988). Also, different responses in plant growth may be a result of different genotypes (MEDHI and HOGAN, 1979).

A slight imbalance in plant growth regulator concentrations at multiplication caused plants to go yellow and produced swollen bases (DREW, 1988). This resulted in poor shoot growth with subsequent loss of vigour and multiplication rate. Recovery from this state was difficult to achieve and plants were normally discarded. The third problem was related to a reversal of form to non-apically dominant shoots at multiplication when plants became abnormally bushy (DE FOSSARD, 1977; DREW, 1992).

### **4.6.3 Rooting and acclimatization**

A major disadvantage of *in vitro* rooting is the difficulty of inducing a root system which will be fully effective when the plants are transferred to soil. Roots produced *in vitro* frequently lack root hairs and vascular connections, and may not begin to develop a secondary cambium until removed from the culture vessel (DEBERGH and MAENE, 1981).

The two phases of adventitious root formation are root initiation, when exposure to auxin is required and root emergence and growth, when exposure to auxin is not required or is inhibitory (WENT, 1939). These two phases are demonstrated *in vitro* with apple rootstocks (JONES *et al.*, 1977). Other authors such as MONCOUSIN (1986) have divided the rooting process into four phases. These include:

- An induction phase, when the capacity for root formation is determined;
- An initiation phase, when visible cytological changes occur;
- An organization phase, when root primordia can be seen to be produced histologically; and
- a growth (root elongation) phase, when primordia develop into roots.

It is important to note here that treatments which promote one of these activities may not be optimal for those which precede or follow.

The major components of the soil environment affecting root growth is moisture, temperature, aeration and fertility (NAMBIAR, 1983). These conditions can be compared to the *in vitro* environment where conditions suitable for growth must be reproduced. Factors known to influence *in vitro* rooting include vessel capacity, media effects (includes micro and macroelements, vitamins, amino acids and sugars), light, temperature and oxygen tension.

ABDULLAH *et al.* (1989) demonstrated the importance of oxygen for root initiation with shoots of *Pinus brutia* which rooted almost as well in aerated tap water as they did on a medium containing growth regulators. No roots were formed unless air was bubbled through the water. In an agar medium there is restricted diffusion. Materials evaluated

for *in vitro* use to provide a porous substrate include coarse sand, autoclaved paper rafts and vermiculite or perlite which will absorb a liquid medium, but allow free diffusion of oxygen. The advantages to using such substrates are higher proportions of shoots forming, root growth is usually better than on agar and roots form root hairs. Another advantage is that the plants can be placed directly into potting medium without the need to rinse any adhering substances from the roots (GEORGE and SHERRINGTON, 1984).

Depending on the treatment received *in vitro*, different kinds of roots are formed and produced. Certain auxins cause the production of a few unbranched roots, while others cause fine, highly branched roots to be produced. Not all kinds of roots ensure plant survival. For example, those produced in the presence of high levels of auxin have an abnormal morphology and function. The application of high concentrations of IBA (higher than 10  $\mu\text{M}$ ) resulted in thickened roots and stunted shoots (DREW, 1988). In *Acer*, shoots similarly rooted at high concentrations of IBA (7.4  $\mu\text{M}$ ) produced abnormally thick, fleshy and fused roots with a reduced ability to survive when acclimatized (McCLELLAND *et al.*, 1990). Any carry-over of small amounts of IBA caused thickened, callused and shorter roots. Also, roots produced in agar had no root hairs, no lateral branches and were of poor quality (DREW pers comm., 1999).

Greater than 90% rooting was achieved using the method described by DREW (1992). DREW *et al.* (1993) reported that although high rooting percentages were achieved with the above nodal culture technique, the plants had small shoots and callused roots. By incubating the plants in darkness for two days on an IBA medium, then transferring the plants to a hormone-free medium supplemented with riboflavin helped to improve the quality of the roots. The problem foreseen in this method was that the shoots were 5 mm in length (a size too small to harden-off as contact with the medium occurs).

RAJEEVAN and PANDEY (1986) obtained the best rooting results on plants subcultured (the fourth subculture) on 20  $\mu\text{M}$  IBA only, however, the shoot growth was stunted and short. RAJEEVAN and PANDEY (1986) point out that the major problem encountered with the use of lateral buds for clonal propagation was the lack of shoot growth concomitant with the development of a normal root system. As mentioned above, apical dominance had to be overcome in such shoots.

RIETVELD (1989) concluded that while plantlet size and shape did not guarantee performance. Optimizing morphological features, such as root: shoot ratio, plantlet size and root system fibrosity could significantly reduce the intensity and duration of transplant stress, thus increasing transplant survival. Likewise, for rooting to occur and for continued growth when transferred from culture, shoots produced during micropropagation must themselves be of good quality and must not deteriorate appreciably during root induction and initiation. Shoot deterioration was thought to be the cause of erratic rooting sometimes reported for *Vitis* (CHEE and POOL, 1988).

Transferring shoots to medium without auxin shortly after a short period of exposure to auxin, is however, impractical. This practice is also not economically beneficial. This resulted in tissue culture laboratories leaving the shoots on the auxin-based medium for longer than three weeks. The resultant roots were thus damaged, with root elongation and functionality affected.

Root formation is generally inhibited after repeated subculture on cytokinin used to induce shoot multiplication or shoot formation (DEFOSSARD, 1977). Sometimes this carry-over effect of cytokinin may inhibit rooting for long periods. In *Albertya magna* where  $1 \text{ mg l}^{-1}$  BA was included, an eight week culture on the root inducing medium was required before rooting commenced (BEN-JAACOV *et al.*, 1991). One of the most important factors for papaya rooting was that the shoots were actively growing when an attempt is made to initiate roots (DREW pers comm., 1999). Steps therefore need to be taken in advance of the rooting stage to reduce the level of cytokinins in shoots. This can possibly be achieved by the absorbing action of activated charcoal. Activated charcoal could also be used to create a darkened environment for the cultures to promote root growth.

Although some authors have reported high rooting percentages in papaya, the roots formed have been poor or the shoot growth has not been sufficient to support growth during acclimatization. Some roots were reported to be callused, thick and lacked root hairs. The continuity of vascular tissue connecting shoot to root was also questionable. If protocols of *in vitro* propagation are to have commercial application to the papaya

industry, then they must consistently produce high rooting percentages and high quality adventitious root systems, which in turn will ensure better acclimatization.

#### **4.6.4 Ethylene production**

Premature senescence of leaves during the culture period remains a major practical problem preventing this technique from becoming commercially established. The closed vessel conditions are known to cause the accumulation of gases in the headspace of the vessel. Such compounds such as ethylene has been identified. PABLITO (1997), using STS (0.3 mM), increased leaf area production and reduced leaf senescence in papaya with nodal culture. The addition of STS to the media (40-50  $\mu$ M) also improved the growth of callus cultures in papaya (ADKINS *et al.*, 1997).

A 0.02-micron filter disc incorporated into the vessel lid can regulate ethylene exchange. However, this option is costly and impractical. LAI *et al.* (2000) reported that an increase in shoot and leaf number was achieved with a regulated exogenous ethylene supply during the first week using a gas diffusion equilibrium procedure. This had to be followed by an aeration of the flasks for the following two weeks. Better control of ethylene and the reduction of culture stress could therefore increase both the quantity and quality of tissue available.

### **4.7 Objectives**

The objectives of this part of the study were to address many of the problem areas described above in order to improve on current micropropagation techniques available and to optimize such techniques in initiation, multiplication and rooting of *Carica papaya* L.

The aims of the studies done on papaya micropropagation were therefore to:

1. determine the effect of sterilizing agents on contamination during the initiation of papaya;
2. determine the effect of activated charcoal on multiplication and rooting of papaya *in vitro*;

3. determine the effect of sucrose concentration on multiplication of papaya;
4. determine the effect of IBA exposure of plantlets on rooting percentage and root type;
5. determine the effect of various supporting media and gelling agents on rooting percentage and root type; and to
6. assess and record a more efficient and practical method of *in vitro* rooting of papaya with better acclimatization potential.

With rooting and acclimatization being problematic areas in the papaya *in vitro* propagation system, a method to overcome both would be to graft unrooted *in vitro* plants to rooted seedlings in the greenhouse. To obtain a superior plant, a selected scion with good fruiting characteristics could be grafted onto a cold tolerant or disease resistant rootstock. Another objective was therefore to look at the effectiveness and practicality of grafting *in vitro* and *ex vitro* with *in vitro* plants.

## CHAPTER 5

# THE EFFECT OF DECONTAMINATING AGENTS AND ANTIBIOTICS ON CONTAMINATION PERCENTAGE OF PAPAYA AT INITIATION

### 5.1 Introduction

As mentioned in Chapter 4, contamination of bud explants from mature papaya trees in the field is one of the most problematic areas in the micropropagation of papaya. Losses of over 80 % have been reported (DREW, 1988). Papaya growth is also known to be hampered by endophytic contamination resulting in lower multiplication rates and reduced plant growth. Endophytic contamination is not immediately visible and may only appear after many subcultures.

Various attempts have been made to reduce the amount of contamination by using smaller explants (DREW and SMITH, 1986), explants from greenhouse grown plants and antibiotics, with varying success. However, this area still requires much improvement as the initiation of cultures is an expensive step and material may be limited for certain selections. Alternative chemicals were chosen in this study to determine their effect on decontamination.

### 5.2 Materials and Methods

#### ***5.2.1 The effect of a Rifampicin dip on the decontamination of papaya explants***

Axillary buds, shoot tips and internodes were taken from papaya seedlings (varieties: Sunrise solo and Tainung) grown in the greenhouse. The explants were initially soaked in a 3 g l<sup>-1</sup> Captan and 0.5 g l<sup>-1</sup> Benlate solution for 30 minutes. The explants were cut further into smaller pieces of approximately 20 mm and the leaves removed. Explants were dipped in 70% ethanol for 10 seconds, then placed into 1% sodium hypochlorite solution for 5 minutes and rinsed twice in sterile distilled water. Half of the explants were placed into a 50 mg l<sup>-1</sup> Rifampicin solution for 20 minutes. All explants were again decontaminated in NaOCl for eight minutes and rinsed three times with sterile distilled

water. The average weight of the explant was 40-80 mg. Explants were placed onto media containing half strength M&S salts, full strength vitamins with 14.5 mg l<sup>-1</sup> L-cysteine, glycine (3,8 mg l<sup>-1</sup>), folic acid (0.88 mg l<sup>-1</sup>), riboflavin (3.8 mg l<sup>-1</sup>), 6% sucrose, 2,4-D and iP (2-20 mg l<sup>-1</sup>) and solidified with 8 g l<sup>-1</sup> agar. The medium had previously been dispensed into glass test tubes and autoclaved for 20 minutes at 121 °C. Cultures were grown in the dark at 25 ± 2°C. Contamination was recorded and contaminated cultures removed three days after initiation.

### **5.2.2 The effect of Bronocide™ on the decontamination of papaya explants**

Explants of papaya were decontaminated using the procedure as described in 5.2.1. Except that Bronocide™ replaced Rifampicin. Explants were also initially divided into small and large pieces based on the stem diameter. Half of the explants were soaked in full strength Bronocide™ (active ingredient: dichlorobenzyl alcohol) for 15 minutes.

### **5.2.3 The effect of various fungicide and antibiotic treatments on the decontamination of papaya explants**

Axillary shoots and buds were taken from mature trees of *Carica papaya* var. Sunrise Solo (AF1) in the field. The shoots were brought to the tissue culture laboratory and all excess leaves and stems removed. The material was soaked or dipped into the following antibiotic and fungicidal treatments:

- 0- No antibiotic treatment
- 1- Rifampicin at 50 mg l<sup>-1</sup>
- 2- Chloramphenicol at 250 mg l<sup>-1</sup>
- 3- Chloramphenicol (250 mg l<sup>-1</sup>) + Rifampicin (50 mg l<sup>-1</sup>) + Streptomycin (200 mg l<sup>-1</sup>)
- 4- TILT® (fungicide) at 0.2 ml l<sup>-1</sup>
- 5- Xanbac®(fungicide) at 2 ml l<sup>-1</sup>
- 6- Benlate® (1 g l<sup>-1</sup>) + Captan® (2 g l<sup>-1</sup>) (fungicide) and 7- No fungicide treatment

Half of the explants were left to soak overnight, while the other half were decontaminated and initiated after one hour soak in the various treatments. The decontamination procedure was followed as described in 5.2.1 using NaOCl as a

decontaminating agent. The Rifampicin dip was replaced by the various treatments listed above. Explants were placed onto medium described in 5.2.1 and incubated in a growth room at  $25 \pm 2$  °C with alternating light/dark 16:8 hour cycles ( $45 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). The number and type of contaminant was recorded after five days.

### 5.3 Results and Discussion

#### 5.3.1 The effect of a Rifampicin dip on the decontamination of papaya explants

Rifampicin at  $50 \text{ mg l}^{-1}$  significantly decreased contamination of papaya explants from the field using the decontamination procedure described, however, contamination still remained higher than 50%. The fungal contaminant *Colletotrichum* was the main fungal contaminant identified.

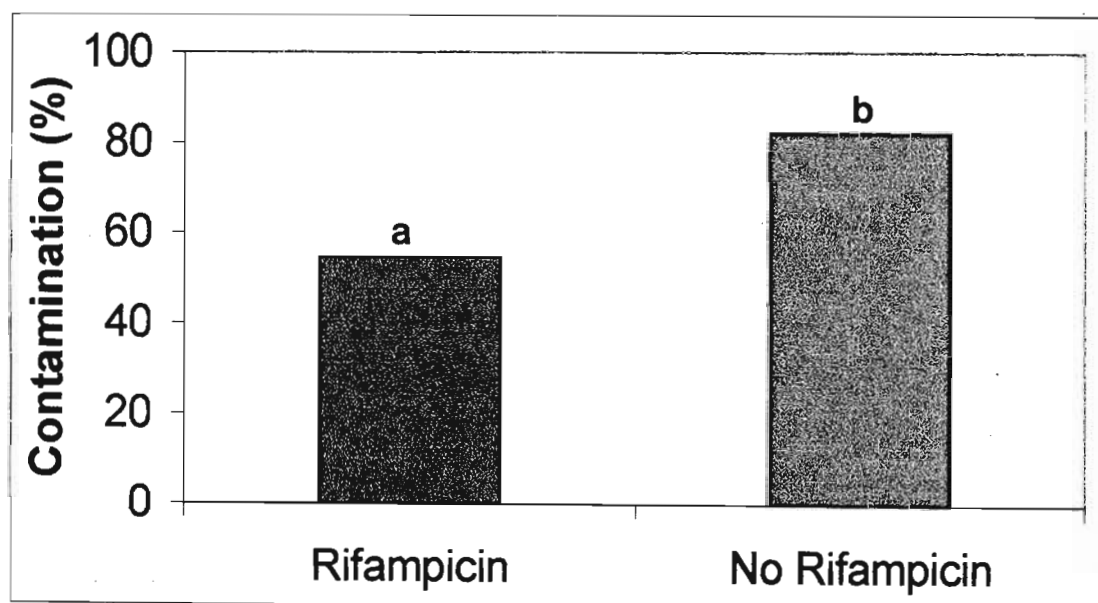


Fig.13: The effect of a 20 minute Rifampicin dip during the decontamination process at initiation on contamination percentage of papaya explants. LSD at 0.05 alpha level = 13.45 (ANOVA). Bars with the same letters are not significantly different.

### 5.3.2 The effect of Bronocide™ on the decontamination of papaya explants

Bronocide™ reduced the contamination for both the small and large explants. Less small explants were contaminated compared to the larger explants. The size of the explant at initiation therefore plays an important role in obtaining clean, uncontaminated tissue (DREW, 1986). The reduced surface area and more effective contact with the decontamination agent in smaller explants possibly played a role. However, no significant differences were obtained between the size of explants and Bronocide™ treatments compared to the number of contaminated plants.

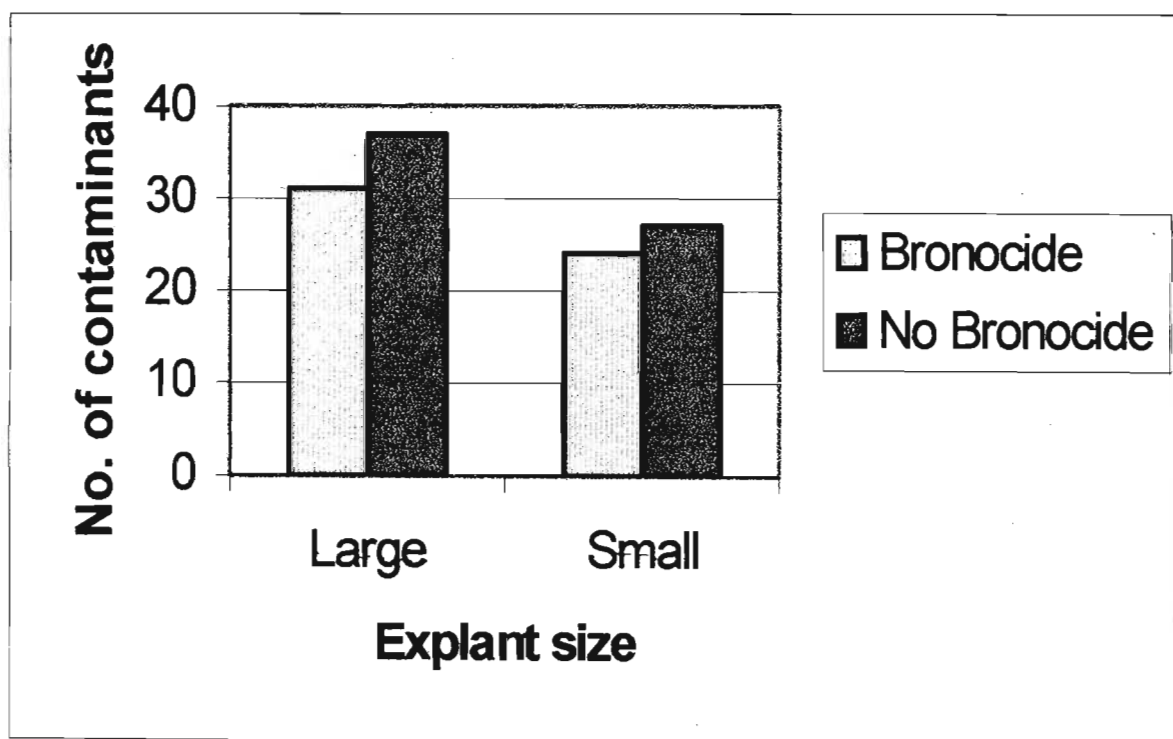


Fig.14: The effect of Bronocide™ treatment and explant size on the contamination percentage of papaya explants (ANOVA Approximate significance: 0.284)

### 5.3.3 The effect of various fungicide and antibiotic treatments on the decontamination of papaya explants

All treatments were more effective in controlling contamination when compared to the control treatment. Percentage contamination was greatly reduced when explants were

soaked in fungicides Xanbac and Benlate+Captan with little or no tissue damage. Although antibiotic treatments Rifampicin and combinations of antibiotics (Rif+Chl+Strep) also reduced overall contamination, there was also a degree of tissue damage. This was possibly due to the concentrations used being too high, thus having a phytotoxic effect.

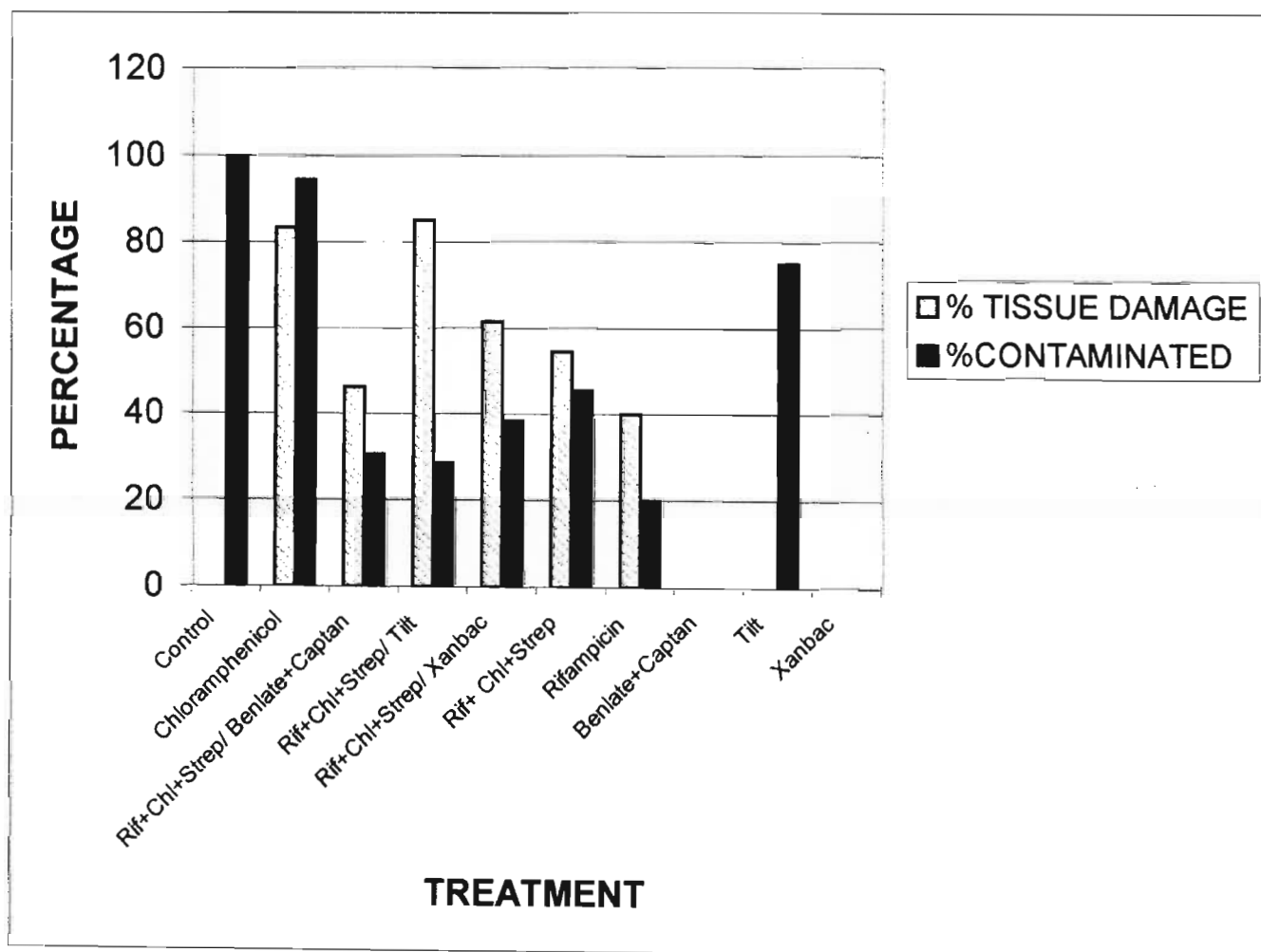


Fig.15: The effect of antibiotic and fungicide treatments on decontamination and tissue damage of papaya explants

#### 5.4 Conclusion

Generally, the pre-soaking or dipping of papaya explants with various decontaminating agents as an extra step during the initiation process had a positive effect on reducing contamination. A presoak of 50 mg l<sup>-1</sup> of the antibiotic Rifampicin and decontaminating agent, Bronocide™, reduced contamination percentage in papaya explants (large

explants) by 28% and 12% respectively. However, although Rifampicin is effective at contamination reduction in explants, the overall cost and the possible negative effects on the plant tissue must be taken into consideration.

Presoaking of explants in fungicide and/ or combinations of fungicides and antibiotics also gave positive results in reducing contamination. Xanbac® and a Benlate®+Captan® combination dip were most effective in overall contamination reduction. TILT® had little effect on contamination control and when used in combination with antibiotics, the tissues were damaged and died. For effective contamination control, Rifampicin and various fungicides should be tested using a range of concentrations for the most effective response.

## CHAPTER 6

# THE EFFECT OF CHARCOAL, GELLING AGENTS, TYPE OF MEDIA, CUTTING AND APPLICATION OF PLANT GROWTH REGULATORS ON THE MULTIPLICATION AND ROOTING OF PAPAYA

### 6.1 Introduction

Many factors influence the multiplication and rooting of papaya plantlets *in vitro*. These include genetic factors such as genotype (NIEDERWIESER and VAN STADEN, 1992), and epigenetic factors such as the type of explant used, apical dominance, size of plant, nutrient medium and environmental conditions.

The aim of this study was to achieve a better and stronger plant during multiplication and to improve root production. Activated charcoal and combinations of gelling and supporting agents were used for rooting. A short outline on the properties of each of these components is given below.

#### 6.1.1 Activated charcoal

Activated charcoal is produced by the destructive distillation of bones, wood, peat and other carbonaceous matter, and the subsequent oxidation of the product in a stream of carbon dioxide. The result is an activated charcoal with superior adsorbing properties, which are due to large specific adsorption areas ( $600$  to  $2000 \text{ m}^2 \text{ g}^{-1}$ ) and pore distributions ( $10$  to  $50 \text{ }\mu\text{M}$ ). The adsorbing properties also are determined by the grade, purity and density of the charcoal, and the pH, inorganic salts and temperature of the media (HALHOULI *et al.*, 1995, PAN and VAN STADEN, 1998).

The addition of activated charcoal to plant growth media is known to have many beneficial effects. These include: the release of naturally present or previously adsorbed compounds into the media (PAN and VAN STADEN, 1998); the creation of a dark environment in the media, thus facilitating the accumulation of photosensitive auxins or co-factors (PAN and VAN STADEN, 1998); the adsorption of inhibitory compounds such as phenolics (WEATHERHEAD *et al.*, 1978) and 5-(hydroxymethyl)-2-furaldehyde

(REDEI, 1974), phenylacetic acid and benzoic acid (FRIDBORG *et al.*, 1978); and the adsorption of plant growth regulators such as cytokinins, auxins and ethylene (EBERT and TAYLOR, 1990; EBERT *et al.*, 1993).

Activated charcoal is reported to preferentially absorb moderately polar compounds rather than highly polar or apolar compounds. Compounds such as glucose, inositol, mannitol and sorbitol which are highly polar may not be adsorbed, while aromatic compounds such as phenolics, cytokinins and auxins are readily adsorbed (PAN and VAN STADEN, 1998).

The rate of adsorption is influenced by the temperature and pH of the media, increasing with high temperatures or low pH values (EBERT and TAYLOR, 1990). The rate of desorption from activated charcoal is very slow. This is dependent upon the grade of the charcoal, the temperature and pH of the solution, and the type of solvent. Desorbed compounds are possibly made available for active uptake by the cultures (PAN and VAN STADEN, 1998).

A number of different species rooted more easily *in vitro* when activated charcoal was added to the medium. Activated charcoal adsorbed auxin so that detrimental effects on root growth were relieved, but it did not do so until the regulant had time to initiate root formation (KRIKORIAN and KANN, 1987). Other factors contributing to the mode of action on improved root growth are: darkening of the culture medium, adsorbing inhibitory substances (preventing tissue blackening) and the slowness in adsorbing auxin, allowing for appropriate uptake as well as affecting differentiation of callus (FRIDBORG *et al.*, 1978).

### **6.1.2 Gelling agents**

Gelling agents are known to influence the growth response of cultures (BORNMAN and VOGELMANN, 1984, GEORGE and SHERRINGTON, 1984; MACRAE and VAN STADEN, 1990; SCHOLTEN and PIERIK, 1998). However, it is normally the cost and availability of the gelling agent that determines the type of material used (DEBERGH, 1983).

Two types of gelling agents were used in this study. The first, Gelrite®, is a gellan gum, which is a water-soluble polysaccharide and is derived from *Pseudomonas elodea*. This polysaccharide is comprised of glucuronate, rhamnose and cellobiose molecules, as well as significant quantities of calcium, magnesium, potassium and sodium (GEORGE and SHERRINGTON, 1984). Although the cost of Gelrite® is higher than that of agar, it is used at lower concentrations and is therefore more cost effective. The effects of the physical properties of Gelrite® in the media are poorly described and studied to date.

Agar is widely used and is also a water-soluble polysaccharide derived from the algae, Rhodophyta particularly *Gelidium* and *Gracelaria* species. The polysaccharide comprises repeating units of  $\beta$ -D-galactose and  $\alpha$ -L-anhydrogalactose, which are frequently substituted with methyl/sulphyl esters of pyruvate ketal groups (WILSON and CRITCHLEY, 1998). The position and quantity of these substitutions influence the chemical and physical properties of the agar. These chemical and physical properties of agar are also influenced by the algal species used (WILSON and CRITCHLEY, 1998), the nutrients (CRAIGIE *et al.*, 1984), the seasons (WHYTE *et al.*, 1981) and the environmental conditions surrounding the algae during their growth (CHRISTELLER and LAING, 1989).

## 6.2 Materials and Methods

*Carica papaya* L. var. "Sunrise Solo" was used in this study. Stock plants were kept in a plastic tunnel with an extraction fan. Plants were watered daily. Initiations were done according to the procedure described in Chapter 5. Sterile plants were multiplied and bulked up for research purposes by subculturing onto fresh media every three weeks. Cultures were incubated under 16:8 light/dark cycles ( $45 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) and maintained at  $25 \pm 2$  °C.

### 6.2.1 *The effect of sucrose concentration on multiplication and growth of papaya*

Papaya multiplication medium for shoot growth was prepared using DREW & SMITH (1986) media (DS) which is a modified DEFOSSARD'S vitamins and salts formulation

(Table 10). Sucrose was added at the following concentrations: 0, 10, 20, 30, 40, 50, and 60 g per litre. The medium was adjusted to a pH of 5.7 with KOH or HCl. The medium was solidified with a gelling agent and hot dispensed into small glass jars with screw lids. The medium was autoclaved at 121°C for 25 minutes (1.1 kg cm<sup>-2</sup>). Papaya plantlets were transferred aseptically into the glass jars. Plantlets were divided according to size (small= 8-10 mm, large= 12-15 mm). An equal amount of each plantlet size group was divided amongst the treatments.

Papaya plants were grown in an environmentally controlled growth room at 25 ± 2 °C with alternating light and dark cycles of 16:8 hours. After four weeks, growth parameters, such as callus production, general appearance, multiplication rate and stem diameter, were measured.

### ***6.2.2 The effect of charcoal on overall growth and multiplication using gelrite as gelling agent***

Media were prepared containing 20 g sucrose, DS salts and vitamins (Table 10), 1 mg NAA and 1 mg BA per litre with the following treatments:

A= 2 g l<sup>-1</sup> gelrite only, B= 7 g l<sup>-1</sup> agar only

C= 1.5 g l<sup>-1</sup> charcoal and 2 g l<sup>-1</sup> gelrite, D= 3 g l<sup>-1</sup> charcoal and 2 g l<sup>-1</sup> gelrite

Media were prepared and cultures incubated as described in section 6.2.1. Ten replications per treatment were used. After four weeks, growth parameters were measured.

Table 10: Papaya multiplication medium (DREW &amp; SMITH, 1986)

Component	Concentration (mg l <sup>-1</sup> )
<u>Macronutrients</u>	
KNO <sub>3</sub>	2020
NH <sub>4</sub> NO <sub>3</sub>	1600
CaCl <sub>2</sub> .6H <sub>2</sub> O	441.1
MgSO <sub>4</sub> .7H <sub>2</sub> O	740
NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	120
<u>Micronutrients</u>	
MnSO <sub>4</sub> .4H <sub>2</sub> O	17.1
ZnSO <sub>4</sub> .7H <sub>2</sub> O	11.5
H <sub>3</sub> BO <sub>3</sub>	9.22
KI	0.83
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.24
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>4</sub> .4H <sub>2</sub> O	1.24
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.24
FeSO <sub>4</sub> .7H <sub>2</sub> O	27.8
Na <sub>2</sub> EDTA	37.22
<u>Growth factors (vitamins)</u>	
Inositol	108.12
Thiamine-HCl	13.492
Nicotinic acid	4.924
Pyridoxine-HCl	1.233
Biotin	0.244
Folic acid	0.882
D-Ca-panthonate	2.382
Riboflavin	3.784
Choline Chloride	1.396
Ascorbic acid	1.761
L-cysteine HCl	14.544
Glycine	3.754

### ***6.2.3 The effect of charcoal on multiplication and rooting using agar as gelling agent***

A multiplication medium was prepared as described above with concentrations of activated charcoal (Sigma grade) added as follows: 0 (control), 1 g l<sup>-1</sup>, 2 g l<sup>-1</sup>, and 3 g l<sup>-1</sup> charcoal. The medium contained no plant growth regulators. The medium was

dispensed into glass test tubes (20 ml/test tube). Autoclave and culture conditions were the same as the above. The plantlets were assessed after four weeks for appearance, rooting response, senescence and multiplication rate.

#### ***6.2.4 The effect of a gelling agent combination on papaya multiplication***

The medium was prepared using DS salts and vitamins as described above and  $0.1 \text{ mg l}^{-1}$  NAA and  $0.1 \text{ mg l}^{-1}$  BA. The supporting agents were added at the following concentrations:

- $1 \text{ g l}^{-1}$  gelrite +  $3.5 \text{ g l}^{-1}$  agar
- $2 \text{ g l}^{-1}$  gelrite (control)

The multiplication rate was calculated as: The total amount of plants obtained in a flask after 4 weeks growth in the growth chamber divided by the initial amount of plants inoculated. Ten replications for each treatment were made (one plant per flask). The multiplication of shoots and appearance of plantlets were assessed after 4 weeks growth.

#### ***6.2.5 The effect of gelling agents on rooting and overall growth of papaya***

Papaya rooting medium I (Table 11) was prepared using method described in 6.2.1. IBA was dissolved in 1 M NaOH, filter sterilized and added to the medium before gelling took place. Plantlets grown previously on multiplication medium were transferred aseptically to PMR 1 medium and grown in the dark at  $25 \pm 2^\circ\text{C}$  for two days.

Papaya rooting II (Table 12) medium was prepared with various concentrations of agar and gelrite. The concentrations applied were 4,5,6,7,8, g of agar-agar and 2 and 3 g of gelrite per litre respectively.

Table 11: Papaya rooting medium I

<b>Component</b>	<b>Concentration (mg l<sup>-1</sup>)</b>
DS Macro and Micronutrients	
<u>Growth factors (vitamins)</u>	
Inositol	108.12
Thiamine-HCl	13.492
Nicotinic acid	4.924
Pyridoxine-HCl	1.233
Biotin	0.244
Folic acid	0.882
D-Ca-panthionate	2.382
Choline Chloride	1.396
Ascorbic acid	1.761
L-cysteine HCl	14.544
Glycine	3.754
Sucrose	20 000
IBA	2
Agar	7 000

Table 12: Papaya rooting medium II

<b>Component</b>	<b>Concentration (mg l<sup>-1</sup>)</b>
DS Macro and Micronutrients	
<u>Growth factors (vitamins)</u>	
Inositol	108.12
Thiamine-HCl	13.492
Nicotinic acid	4.924
Pyridoxine-HCl	1.233
Biotin	0.244
Folic acid	0.882
D-Ca-panthionate	2.382
Choline Chloride	1.396
Ascorbic acid	1.761
L-cysteine HCl	14.544
Glycine	3.754
Riboflavin	3.784
Sucrose	20 000
Agar	27 000

Table 13: Papaya elongation medium (PEG)

<b>Component</b>	<b>Concentration (mg l<sup>-1</sup>)</b>
<u>Macronutrients</u>	
KNO <sub>3</sub>	2020
NH <sub>4</sub> NO <sub>3</sub>	1600
CaCl <sub>2</sub> .6H <sub>2</sub> O	441.1
MgSO <sub>4</sub> .7H <sub>2</sub> O	740
NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	120
<u>Micronutrients</u>	
MnSO <sub>4</sub> .4H <sub>2</sub> O	17.1
ZnSO <sub>4</sub> .7H <sub>2</sub> O	11.5
H <sub>3</sub> BO <sub>3</sub>	9.22
KI	0.83
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.24
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>4</sub> .4H <sub>2</sub> O	1.24
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.24
FeSO <sub>4</sub> .7H <sub>2</sub> O	27.8
Na <sub>2</sub> EDTA	37.22
<u>Growth factors (vitamins)</u>	
Inositol	108.12
Thiamine-HCl	13.492
Nicotinic acid	4.924
Pyridoxine-HCl	1.233
Biotin	0.244
Folic acid	0.882
D-Ca-panthionate	2.382
Riboflavin	3.784
Choline Chloride	1.396
Ascorbic acid	1.761
L-cysteine HCl	14.544
Glycine	3.754
<u>Plant growth regulators</u>	
Kinetin	10
NAA	5
GA3	5
Adenine-sulphate	800

Plantlets previously grown in the dark on papaya rooting medium I were transferred aseptically on a laminar flow bench to the jars containing the various media. The papaya plants were grown at  $25 \pm 2$  °C in the light (8 hours off and 16 hours on) for four weeks. For each treatment there were 10 jars with 4 plants per jar. After four weeks, general appearance and rooting percentages were recorded. Random samples of rooted plantlets were taken and viewed under light and dissecting microscopes respectively. Stomata samples were prepared using clear tape to remove the abaxial layer. Stains were made using iodine for the stomata and phloroglucinol-HCl for vascular tissue examination of papaya roots.

### ***6.2.6 The effect of IBA exposure with combinations of charcoal and low agar and gelrite concentrations on rooting***

Media were prepared according to the following treatments:

A: 2 g gelrite + 2 mg l<sup>-1</sup> IBA, and DS salts (vitamins omitted)

B: 4 g agar + 2 mg l<sup>-1</sup> IBA, and DS salts (vitamins omitted)

C: 2 g l<sup>-1</sup> gelrite + 2 mg l<sup>-1</sup> IBA + 2 g l<sup>-1</sup> charcoal + DS salts and vitamins

D: 4 g l<sup>-1</sup> agar + 2 mg l<sup>-1</sup> IBA + 2 g l<sup>-1</sup> charcoal + DS salts and vitamins.

The media were sterilized and dispensed into sterile glass bottles after the addition of IBA through a 4.5 micron filter. The plants were placed into a growth chamber at  $25 \pm 2$  °C in the dark. After two days, treatments C and D were transferred to a growth chamber of  $25 \pm 2$  °C with a 16 hour light and 8 hour dark cycle.

The plants from treatments A and B were transferred to the following media:

E: 4 g l<sup>-1</sup> agar + DS salts and vitamins without IBA

F: 2 g l<sup>-1</sup> gelrite + DS salts and vitamins without IBA

G: 4 g l<sup>-1</sup> agar + DS salts and vitamins + 2 g l<sup>-1</sup> charcoal, without IBA

H: 2 g l<sup>-1</sup> gelrite + DS salts and vitamins without IBA

Two plants were cultured per bottle, with nine bottles per treatment. The number and length of roots, the type of root and overall appearance of the plantlet were recorded after four weeks.

### **6.2.7 The effect of PEG, charcoal and L- cysteine with vermiculite as a supporting medium on root type**

IBA (5 mg) was filter sterilized into one litre of sterile water. Half of the solution was decanted into a 500 ml sterile glass beaker. Papaya plantlets were removed from the multiplication medium, cut and placed in the solution (primed) for one hour on the laminar flow bench. Only the stems were submerged in the liquid. Two plants were placed into glass bottles containing 40 ml dry vermiculite and 40 ml of the following:

- 1) DS salts and vitamins- control
- 2) 1 g l<sup>-1</sup> PEG 6000 with DS salts and vitamins
- 3) 3 g l<sup>-1</sup> PEG 6000 with DS salts and vitamins
- 4) Added L-cysteine (60 mg l<sup>-1</sup>) with DS salts and vitamins
- 5) 3 g l<sup>-1</sup> PEG 6000 with 3 g l<sup>-1</sup> charcoal and DS salts and vitamins.

Cultures were incubated in light/dark cycles of 16:8 hours in a growth chamber regulated at 25 ± 2°C. After four weeks, percentage rooting, type of root, and general appearance of the plantlets were recorded for each treatment.

### **6.2.8 The effect of cutting and callus growth on multiplication**

Papaya was subcultured from papaya elongation medium (Table 13) onto papaya multiplication medium. On some cultures, the callus was removed from the base of the plant, while other plants were not cut and the callus was kept intact at the base of the plant. The plants were transferred into 100 mm glass jars containing six plants per jar with five jars per treatment. Plants were incubated under the same conditions described above. Overall appearance and multiplication rate was recorded after four weeks.

### **6.2.9 The effect of callus removal on elongation of papaya**

Six papaya plantlets were cultured into each bottle containing Papaya elongation medium (Table 13). For half the plantlets, the callus was removed from the base of the plant, while for the remaining plantlets, the callus was retained with no cutting. The length of the plants was marked on the sides of the bottles at inoculation. The plantlets

were incubated under the conditions described above for four weeks. The difference in plantlet length after four week's growth was measured and recorded.

### **6.2.10 Grafting of papaya**

#### **6.2.10.1 Grafting *in vitro***

Rooted plants in agar were used for this trial. Smaller plants from the multiplication stock were used for the scion stock. Stretchable bandage (STERICREPE, from Northampton, England) was autoclaved in its packaging in a glass container for 20 minutes at 121 °C. On a laminar flow bench, a slanted cut was made removing the rooted plant shoot tip, while on the scion material, the callus base was removed with a matching slanted cut. The bandage was cut to size and wrapped around the graft twice. The grafted plantlet was then placed into vermiculite medium in a glass container.

The above procedure took about 10 minutes and was difficult to execute as the tissue culture material was very slippery and the cuts difficult to hold together.

#### **6.2.10.2 Grafting *ex vitro***

Seeds from *Carica papaya* var "Sunrise Solo" were planted in one litre bags with vermiculite, peat and pine bark (1:1:1) in volume as a substrate. Seedlings grew in a greenhouse under a mist irrigation system until established (15 cm in length). Plants multiplied *in vitro* without roots were used as scions for the graft. Wedge and slant grafting techniques were applied. Grafting tape, masking tape and Parafilm™ were used to fuse the graft. Ten replications were made for each treatment. Forty days later the rate of success was recorded. Some plants were also fused using a small plastic peg, which was quicker to apply.

### ***6.2.11 The effect of modified lids with cotton-wool plugs on senescence***

Papaya plants (all uniform, but light in colour) from multiplication media, were dipped into a 5 mg l<sup>-1</sup> IBA solution for two hours. The plants were removed from the solution and placed into glass flasks (100mm in height), containing the following treatments:

- 1) DS salts and vitamins,
- 2) DS salts and vitamins (with cotton-wool plugs in the lids),
- 3) DS salts and vitamins with PEG 6000 (3 g l<sup>-1</sup>),
- 4) and DS salts and vitamins with 6 g l<sup>-1</sup> activated charcoal.

The solutions were buffered to a pH of 5.7 and then added in equal volumes to vermiculite and autoclaved at 121 °C for 25 minutes. The plants were incubated as above and leaf abscission recorded after four weeks in culture.

## **6.3 Results**

### ***6.3.1 The effect of sucrose concentration on multiplication and growth of papaya***

All treatments, except for a sucrose concentration of 20 g l<sup>-1</sup> were significantly different to the control (Fig. 16). Sucrose is therefore important for overall multiplication of the plantlets. Sucrose added at 50 g l<sup>-1</sup> into the medium gave the best plant multiplication rate. It is important to note here that no plant growth regulators were added. This was done to determine the full effect of the sucrose alone on multiplication. The higher the sucrose concentration, the higher the callus production observed. Callus was formed at the base of the plant.

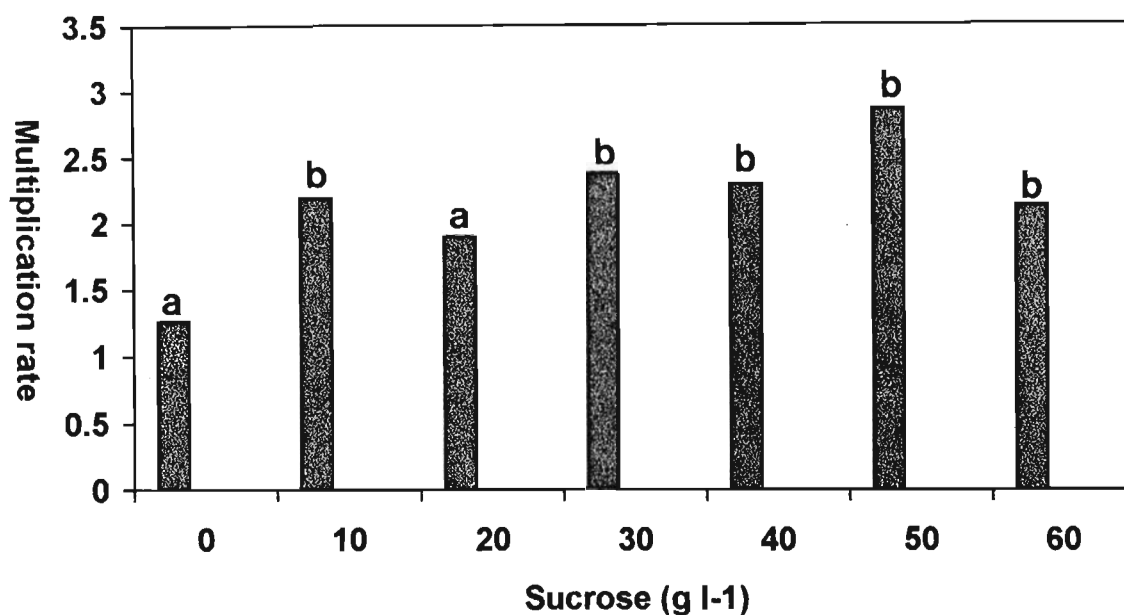


Fig. 16: The effect of sucrose concentration on multiplication rate of papaya.

LSD at 0.05 alpha level=0.766. Bars with the same letters are not significantly different at 0.05 alpha level.

At lower sucrose concentrations, the leaves were lighter green and small with extended petioles and the veins were pronounced. Those plants grown on higher sucrose concentrations *in vitro* were greener.

### **6.3.2 The effect of charcoal on overall growth in multiplication using gelrite as a gelling agent**

The multiplication rates of plants cultured on gelrite only and gelrite with a low concentration of activated charcoal were significantly higher than plants cultured on agar and gelrite + higher concentrations of activated charcoal (Fig. 17)

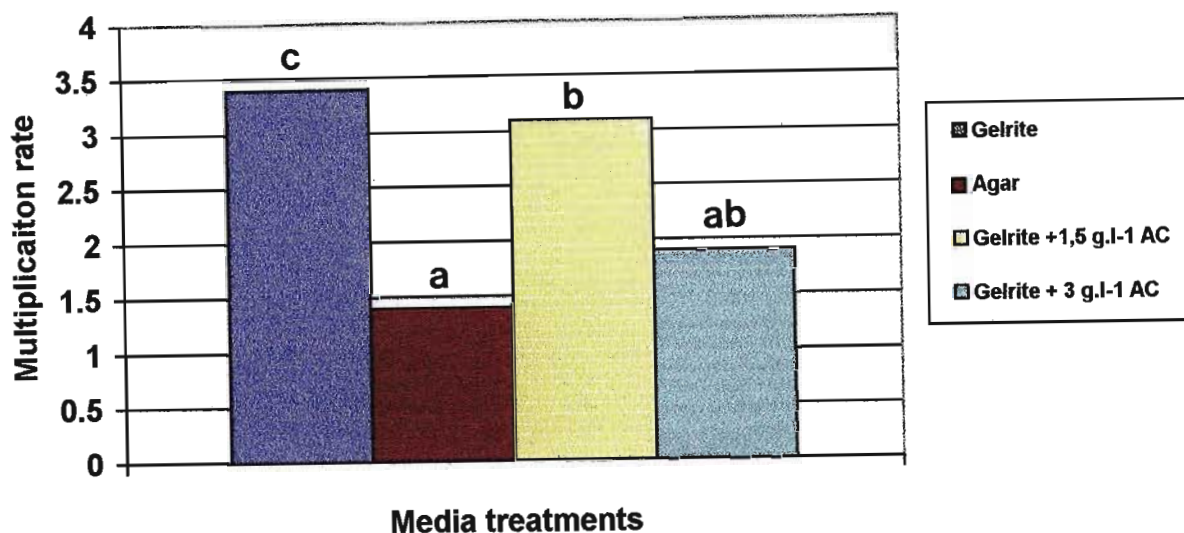


Fig. 17: The effect of gelling agent and activated charcoal concentration on papaya multiplication rate *in vitro*. LSD at 0.05 alpha level = 1.316. Bars with the same letters are not significantly different at 0.05 alpha level.

Table 14: The effect of activated charcoal and type of gelling agent on papaya vitrification and callus development

Gelling agent	Charcoal concentration	Callus development	Vitrification
2 g l <sup>-1</sup> gelrite	0	100%	100%
7 g l <sup>-1</sup> agar	0	10%	10%
2 g l <sup>-1</sup> gelrite	1.5 g l <sup>-1</sup>	100%	100%
2 g l <sup>-1</sup> gelrite	3 g l <sup>-1</sup>	0%	0%

Gelrite increased multiplication of the plants *in vitro*. However, vitrification occurred which made further multiplication difficult. Activated charcoal, added at a concentration of 1.5 g l<sup>-1</sup>, was not sufficient to reduce the vitrification effect on the plants. Activated charcoal, supplemented at a concentration of 3 g l<sup>-1</sup>, did have an effect on reducing

vitrification, however, the multiplication rates were lower. The vitrification effect on plants cultured on gelrite + lower concentrations of activated charcoal was lower than those cultured on gelrite alone.

### **6.3.3 The effect of charcoal on multiplication and rooting using agar as gelling agent**

Since most of the above trial was contaminated, no statistical analysis could be done and only observations were recorded from the remaining material. The higher the concentration of activated charcoal added to the medium, the greener the plant. This was also observed in other experiments. Activated charcoal also appeared to have an effect on overall senescence by reducing the negative effects of leaf drop (Table 15). Of interest here is that rooting occurred on medium that was supplemented with 2 g l<sup>-1</sup> activated charcoal. Roots formed were long, smooth and white in appearance (Fig. 18A). It is possible that with an increasing concentration of AC, the AC adsorbed toxic compounds in the medium, accounting for better growth.

Table 15: The effect of activated charcoal concentration on the appearance of papaya *in vitro*

<b>AC concentration (g l<sup>-1</sup>)</b>	<b>Appearance of plant</b>	<b>Senescence</b>
0	Veined, spindly leaves	Yes
1	Good plants, light yellow	No
2	Green leaves	No
3	Dark green leaves, good plants	No

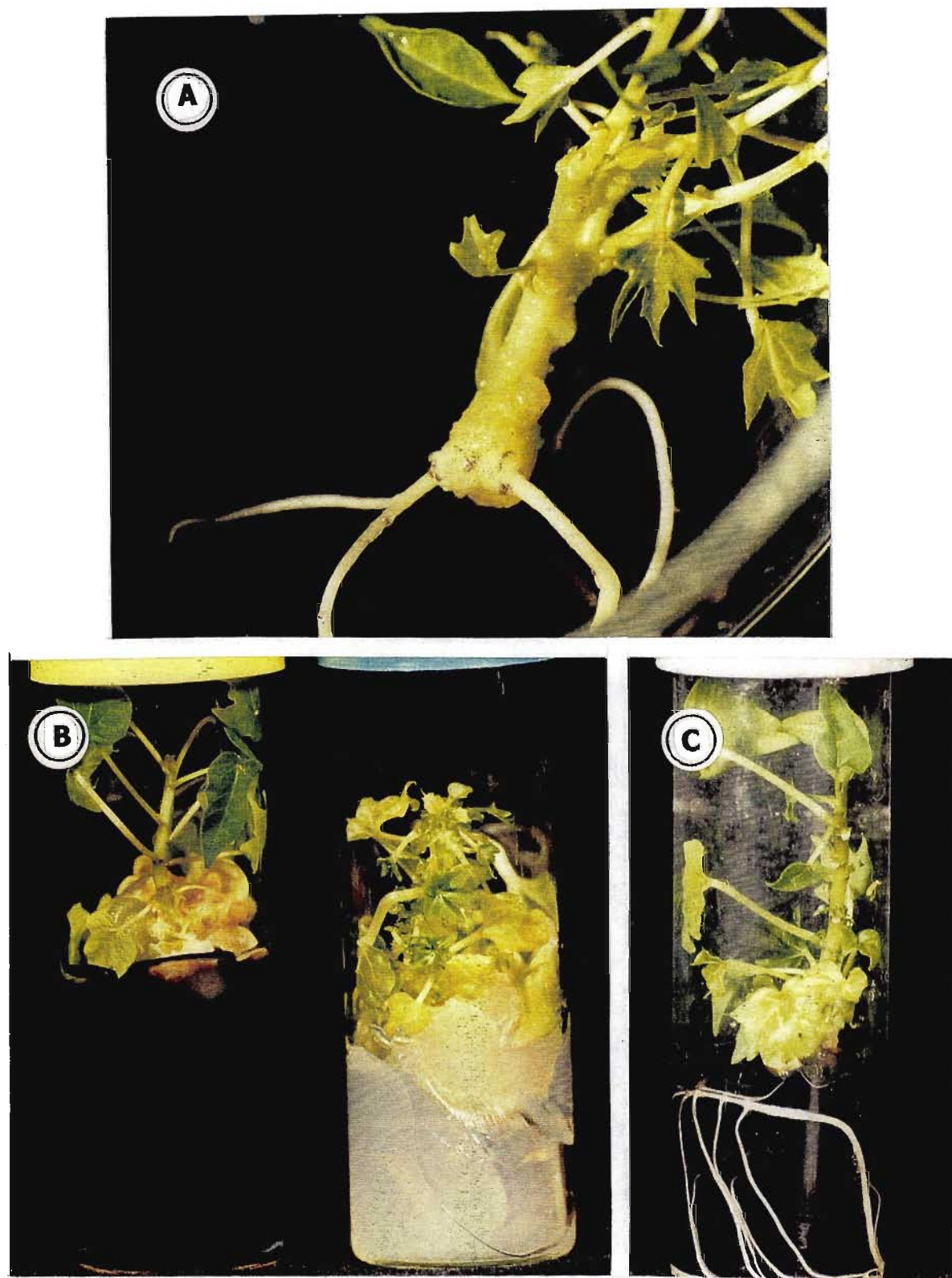


Fig.18A:Smooth, white roots of papaya formed on medium containing activated charcoal; B: Papaya plantlets grown with (left) and without (right) activated charcoal; C: rooting of papaya on medium supplemented with 2 g l<sup>-1</sup> activated charcoal

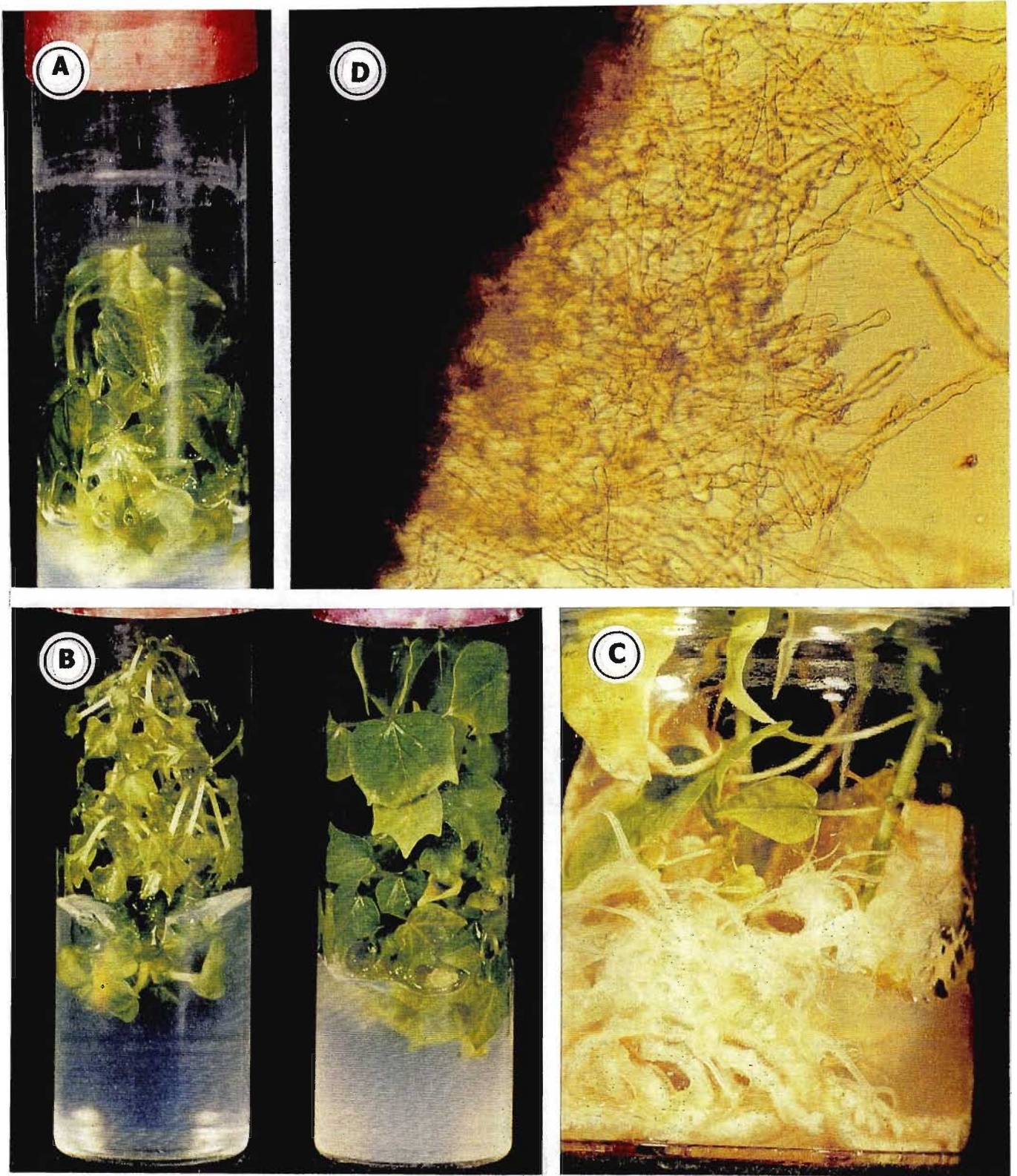


Fig. 19A: The vitrification of papaya shoots when cultured on a medium solidified with Gelrite; B: Papaya plantlets cultured on a medium containing Gelrite (left) showing extended petioles and vitrified shoots, and Agar+Gelrite (right), with good shoot development; C: Thick callused roots of papaya formed in agar medium after two months in culture on an IBA rooting medium; D: Root hair formation on a root of papaya *in vitro*

#### **6.3.4 The effect of a gelling agent combination on multiplication rate of papaya**

The multiplication rate of papaya *in vitro* grown on a medium solidified with gelrite was much higher than other treatments (Table 16). However, as shown in previous trials (6.3.2), vitrification occurs (Fig. 19A) thus making further multiplication difficult. Gelrite alone at the concentration used should therefore not be used as a solidifying medium for the multiplication of papaya *in vitro*. The combination of agar and gelrite does have a positive effect on vitrification elimination (Fig. 19B), but there is a compromise on the multiplication rate of the plants. The gelling agent combination appears to be a better option than agar alone (see 6.3.2).

Table16: The effect of an agar and gelrite combination on vitrification and multiplication rate of papaya *in vitro*.

<b>Treatment (per litre)</b>	<b>Multiplication rate</b>	<b>Vitrification observed</b>
Agar (3.5 g)+Gelrite (1 g)	2.9	No
Gelrite (2 g)	4.7	Yes

#### **6.3.5 The effect of gelling agents on rooting and overall appearance of papaya**

The highest rooting percentage achieved for papaya plants was recorded when they were grown on a low concentration of gelrite (2 g l<sup>-1</sup>) (Fig. 20). However, if kept in culture for longer than three weeks, the plants became vitrified. There appears to be no significant difference in the concentration of agar used and the rooting percentage (Fig. 21). However, differences in growth between the treatments was visible, with the plants grown in the lowest concentrations being greener and stronger than those grown in higher concentrations of the gelling agents. Root growth was also better with the lower concentrations of gelling agents.

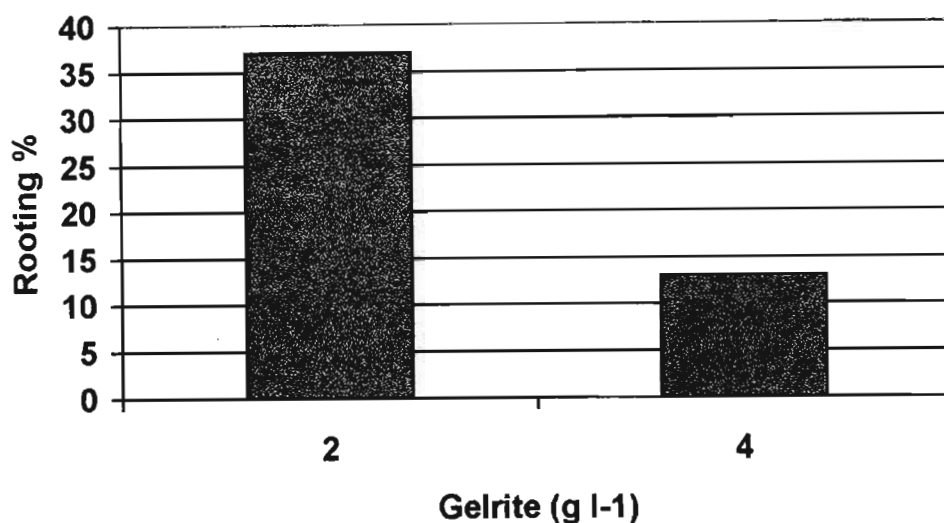


Fig. 20: The effect of Gelrite concentration on the rooting percentage of papaya *in vitro*.

Roots formed on agar medium tended to be thick and callused (Fig. 19C), especially if left on a medium containing plant growth regulators as well. The stomata on the leaves of plantlets cultured on agar (Fig. 22A) and Gelrite (Fig. 22B) showed open guard cells, thus allowing much transpiration.

The type of root produced is important in the acclimatization stage. The development of lateral roots and root hairs (Fig. 19D) is beneficial for survival. Aeration in the rooting medium is beneficial to the development of such roots *in vitro*. Low concentrations of agar and gelrite, although providing a little aeration, did not provide the required support for the plants to stand upright in the vessel. The plants from the lower concentrations of gelling agents tended to topple over in the flask. Also, the plants grown in gelrite (especially at the higher concentrations) were vitrified. Should gelrite therefore be used, it would have to be at lower concentrations for shorter culture periods or in combination with agar or other gelling or supporting agents. Hence, it was decided to also look at other supporting media such as vermiculite and lower

concentrations of gelling agents with activated charcoal to reduce the degree of vitrification.

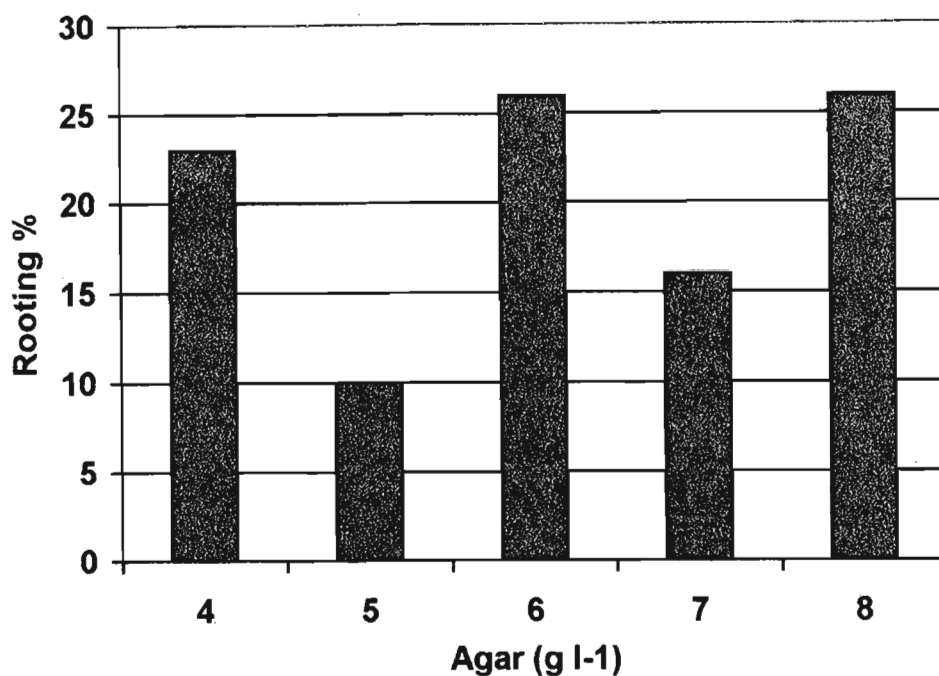


Fig. 21: The effect of agar concentration on rooting percentage of papaya *in vitro*.

### **6.3.6 The effect of IBA treatments with combinations of charcoal and low agar and gelrite concentrations on rooting**

The type of root formed was affected by the addition of activated charcoal in the medium. Those roots grown in the presence of activated charcoal were smoother and white in appearance, while those formed in the absence of activated charcoal in the medium were callused and thick (inferior roots). The addition of 2 g l<sup>-1</sup> activated charcoal did not help in counteracting the negative effects of long exposure to IBA in the medium. Rooting percentage was greatly reduced (Table 17) and plants generally showed poor growth (vitrified). A pulse of IBA for two days was far superior to a 28 day continuous treatment.

The number and length of roots (Table 17) were greater for those plants grown in agar than in gelrite (Fig. 23A). Multiple shoot formation was greater on plantlets grown on gelrite than on agar. For the rooting stage, this is not desirable (Fig. 23B).

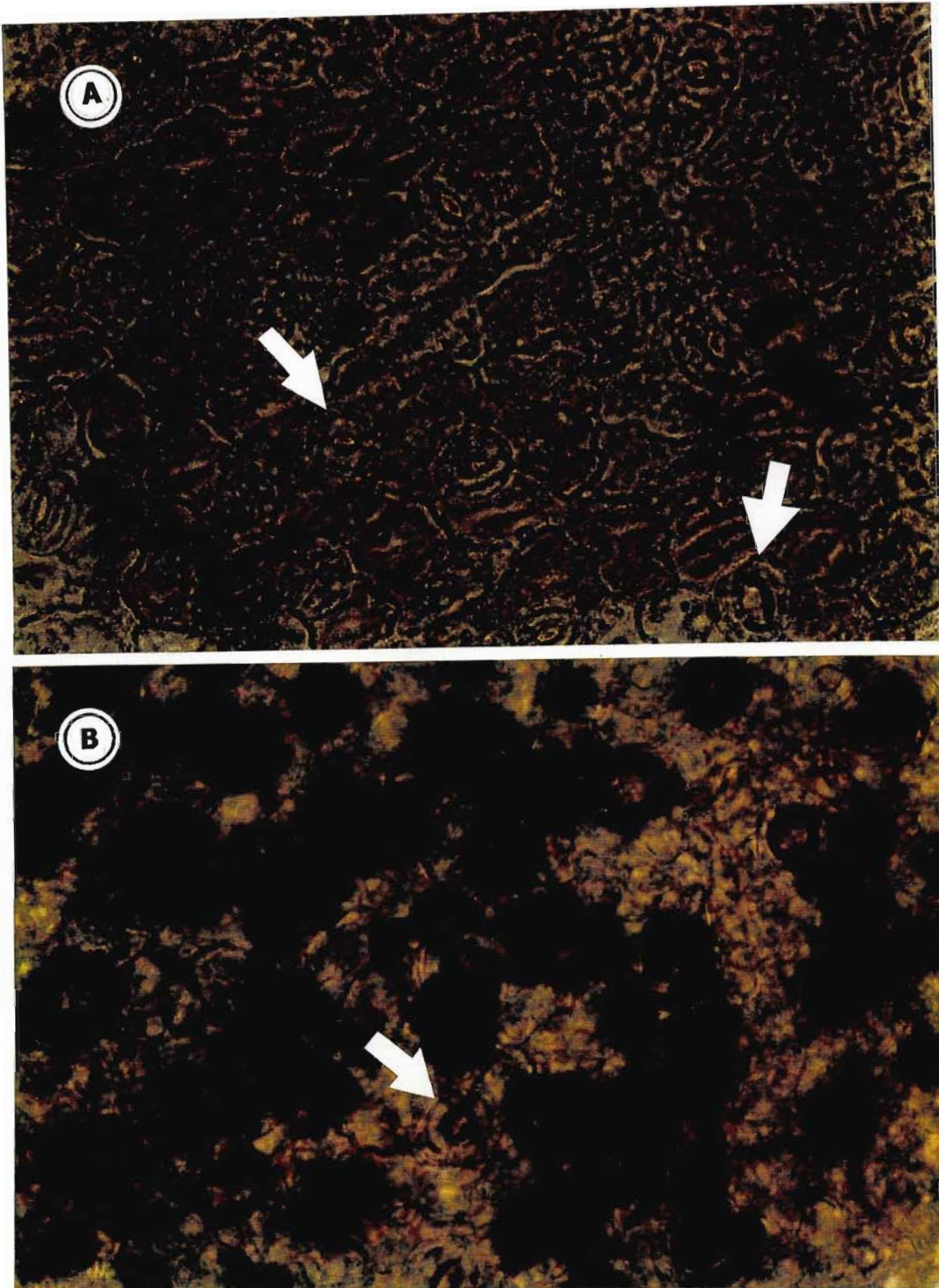


Fig. 22A: Stomata of papaya cultured on agar and B: on Gelrite (Mag x 400)

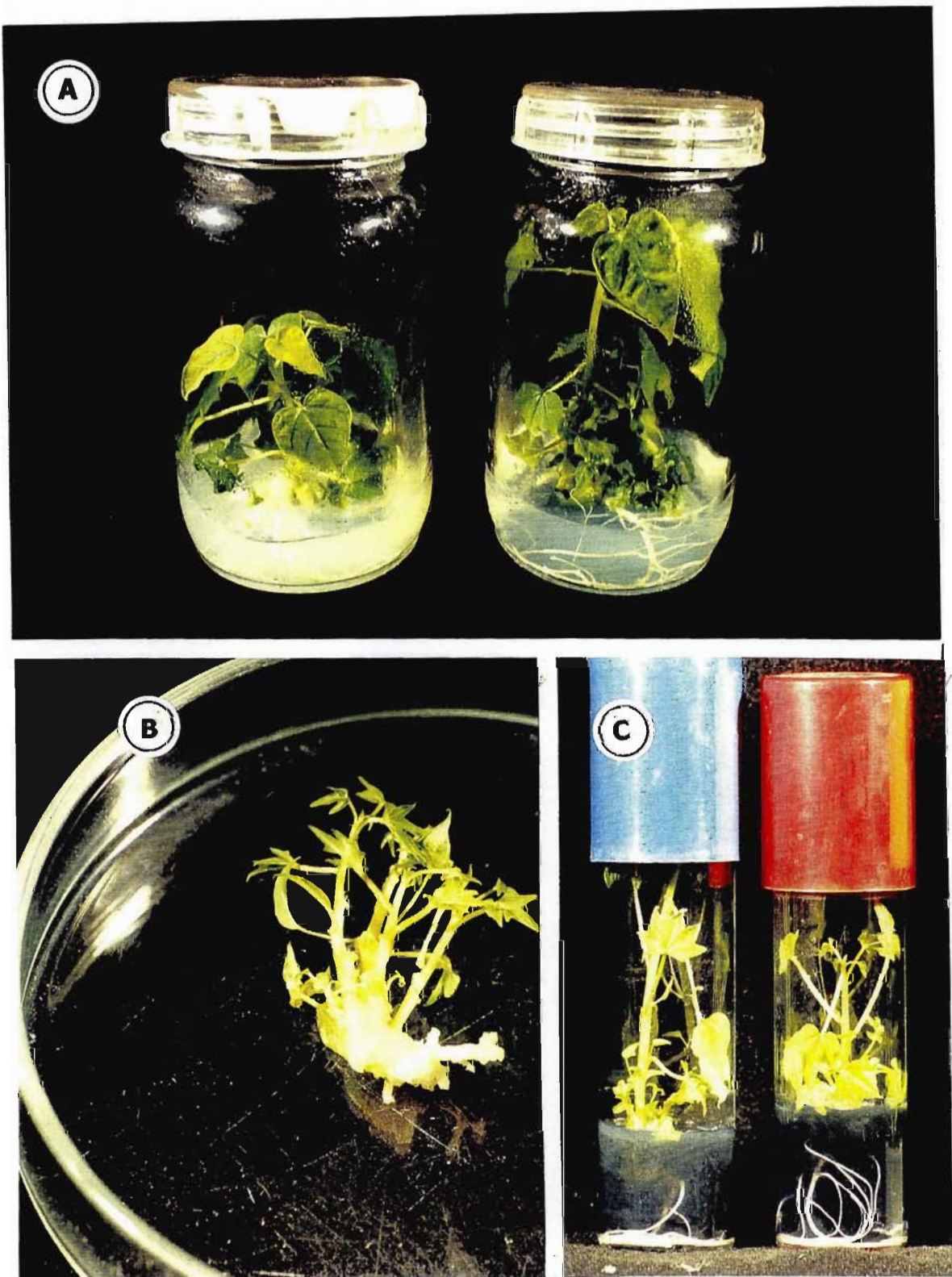


Fig.23A: Papaya plantlets cultured on agar (left) and Gelrite (right) showing lateral roots; B: Multiple shoot formation of a papaya plantlet *in vitro* cultured on a medium supported with Gelrite; C: Smooth, white roots of papaya *in vitro* formed on medium supplemented with 0.3 % activated charcoal

The roots formed on medium supplemented with activated charcoal were thin, white and smooth in appearance (Fig. 23C).

Table 17: The effect of IBA exposure, gelling agent and activated charcoal on overall appearance and type, number and length of roots of papaya *in vitro*.

Gelling Agent (g l <sup>-1</sup> )	IBA treatment (days)	AC (g l <sup>-1</sup> )	Type of root	Appearance	Ave. no. of roots	Ave. length of roots (cm)
2 Gelrite	28	2	Large, smooth	Dark green	0.0625	0.185
4 Agar	28	2	0	Vitrified	0	0
4 Agar	2	0	Callused, laterals	Green, elongated	3.5	4.56
2 Gelrite	2	0	Callused, stem laterals	Multiple shoots	2.25	4.4325
4 Agar	2	2	Smooth, off callus	Green, good	3.2	3.2
2 Gelrite	2	2	Smooth, laterals	Elongated, good	2.45	1.875

Overall, the plants grown on 4 g l<sup>-1</sup> agar, without activated charcoal and only pulsed with IBA for two days showed a significantly higher rooting percentage than those plants treated with IBA for 28 days and the plants grown on gelrite with activated charcoal + a two day IBA pulse. Overall the rooting percentage was significantly higher in plants pulsed for two days than those treated for 28 days with IBA.

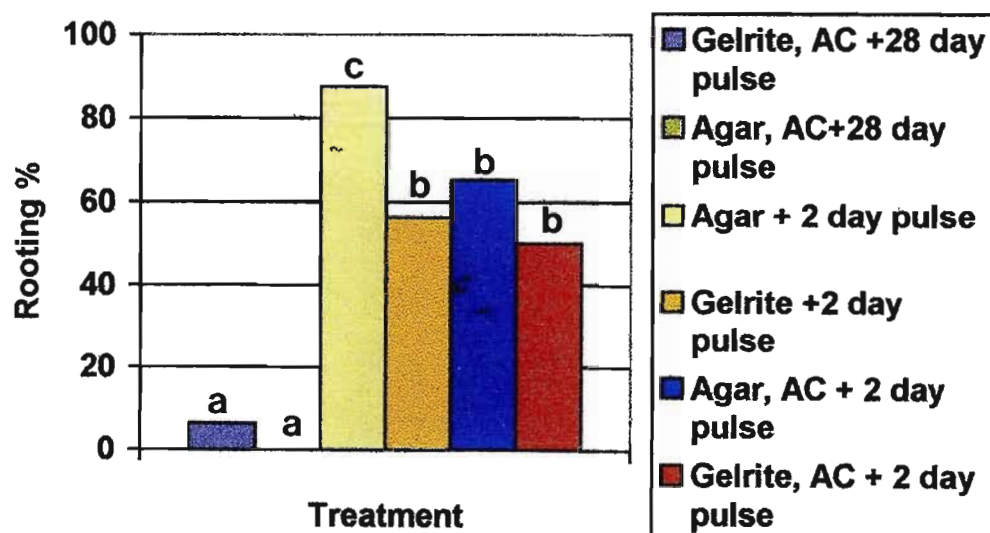


Fig. 24: The effect of gelling agent, activated charcoal and IBA exposure treatments on rooting percentage of papaya *in vitro*. LSD at 0.05 alpha level= 33.05

### 6.3.7 The effect of PEG, charcoal and L- cysteine with vermiculite as a supporting medium on root type

The highest rooting percentage was achieved with plantlets treated with PEG + activated charcoal, PEG alone (3 g l<sup>-1</sup>) and the control (Table 18). The addition of activated charcoal to the PEG treatment did not appear to have a negative or positive effect on rooting percentage. This is possibly due to the activated charcoal precipitating to the bottom of the vessel, and thus not being evenly distributed enough for an effect to occur. No difference in rooting percentage was found whether PEG or L-cysteine was added to the vermiculite medium or not.

Table 18: The effect of various osmotica and activated charcoal on rooting percentage of papaya in vermiculite. LSD at 0.05 alpha level = 46.081 (ANOVA), NS in table indicates Non-significance at 0.05 alpha level on rooting percentage.

Code	Osmotica (g l <sup>-1</sup> )	Activated charcoal (g l <sup>-1</sup> )	Rooting (%)	Root type
1	None	0	80 NS	Good, strong roots
2	PEG 6000 (1 g l <sup>-1</sup> )	0	40 NS	Many roots
3	PEG 6000 (3 g l <sup>-1</sup> )	0	80 NS	Small initials
4	L-Cysteine (0.06 g l <sup>-1</sup> )	0	75 NS	Longer roots
5	PEG 6000 (3 g l <sup>-1</sup> )	3	80 NS	Numerous roots

After four weeks, small root initials were seen at the base of the plant and around the stem in some treatments (Fig. 25B), while long, strong roots were seen on plants in other treatments (Fig. 25A, Fig. 25C). Root hairs could be seen without a microscope on all roots formed in the vermiculite medium.

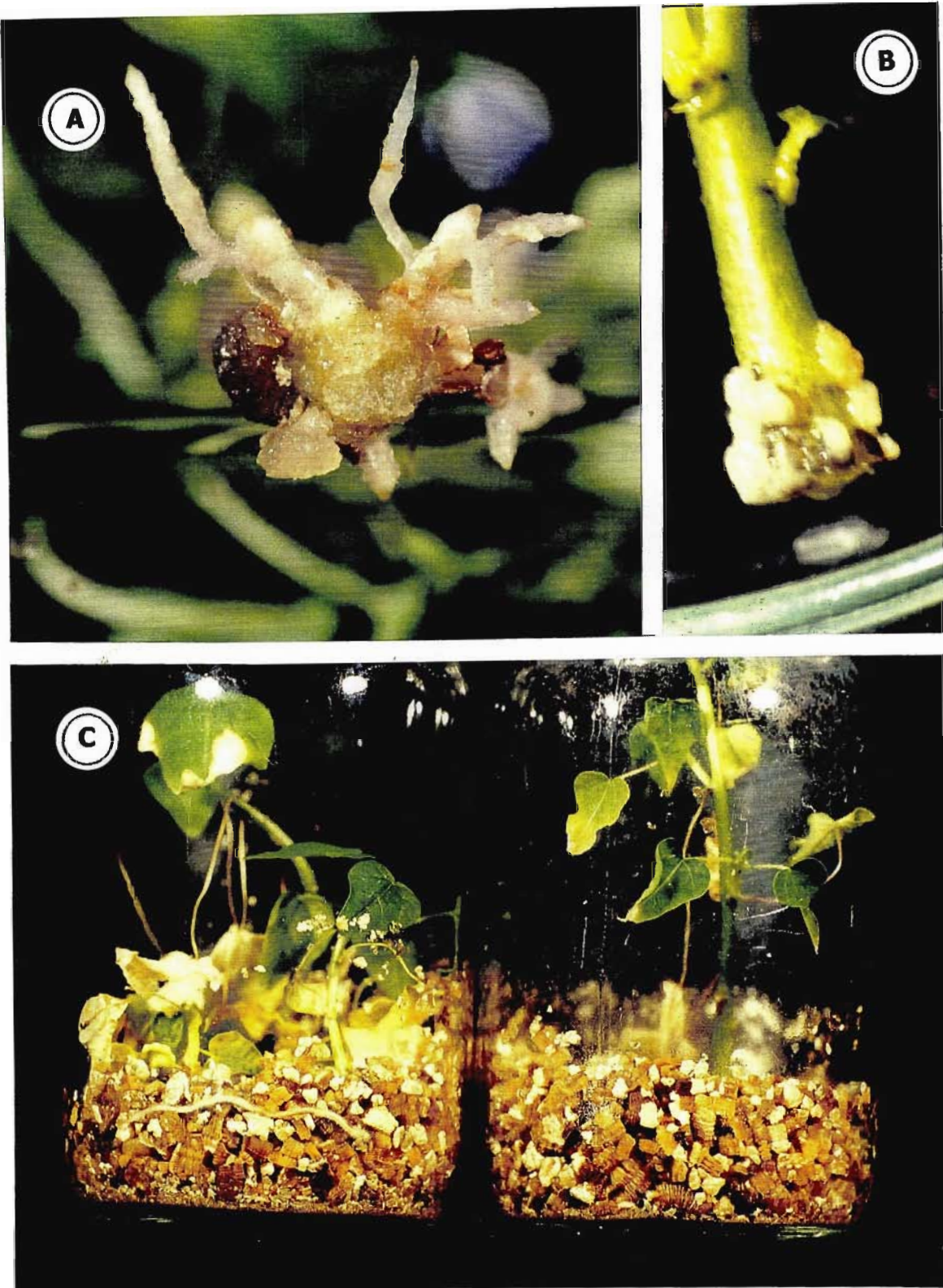


Fig.25A:Roots of a papaya plantlet rooted on vermiculite; B: Root initials forming at the base of papaya *in vitro* rooted on vermiculite; C: Root formation of papaya *in vitro* within vermiculite

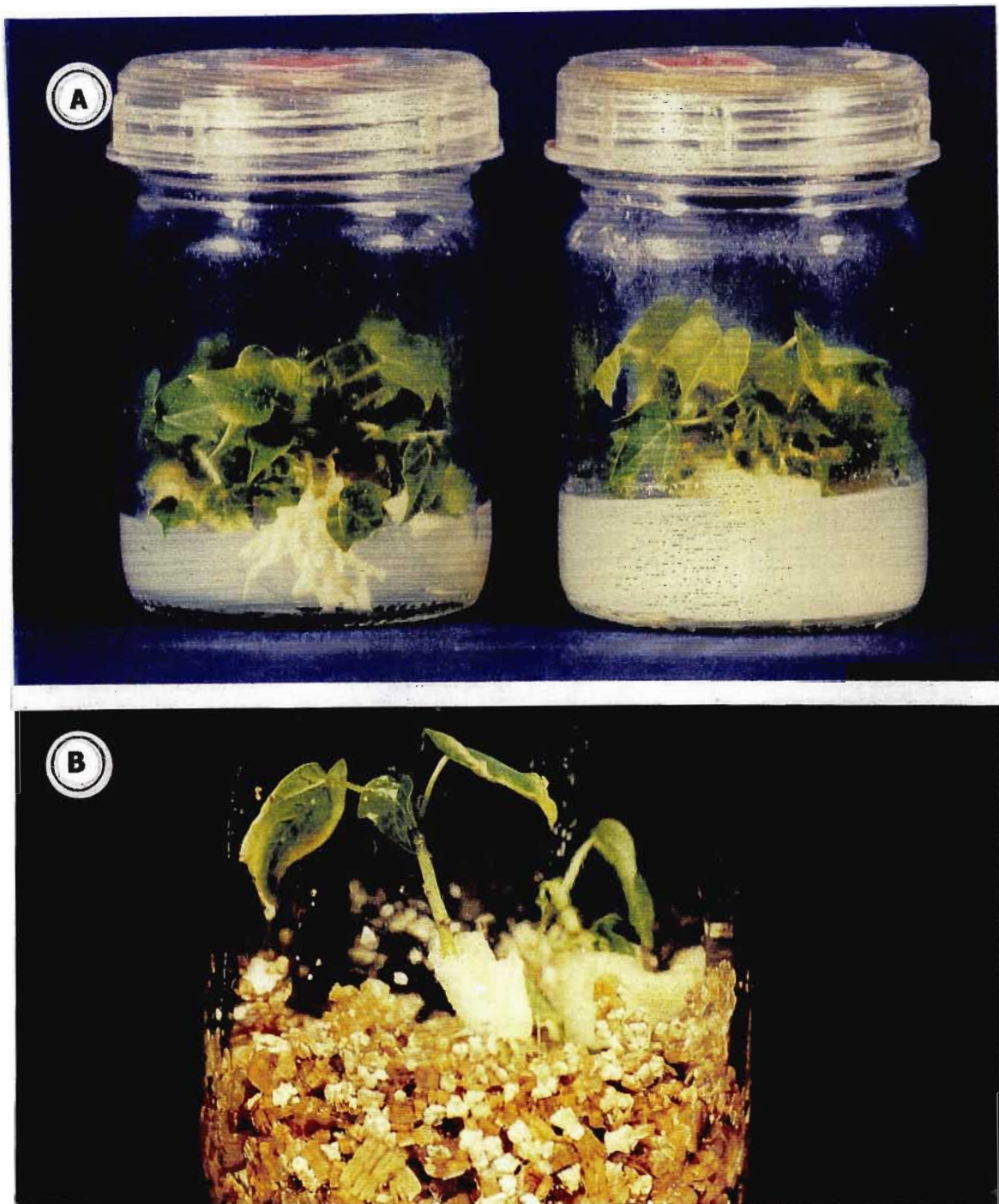


Fig.26A: Multiplication of papaya shoots with callus retained at the base (left) and root formation in agar *in vitro* (right); B: Papaya plantlet grafted with sterile crepe bandage *in vitro*

### 6.3.8 The effect of callus removal from the bases of papaya plantlets on *in vitro* multiplication

Plants transferred with callus at the base of the plantlets (Fig. 26A) clearly showed better growth after four weeks than plants where the callus had been removed. For papaya plantlets with callus retained at the base of the plant, elongation was much improved. Axillary bud areas were also red and the plant appeared more typical of a mature papaya. Plantlets where the callus had been removed were small, short and vitrified. Plants placed on multiplication media with callus achieved an average multiplication rate of 2.1, significantly different to the plants with the callus removed with a multiplication rate of 1.5.

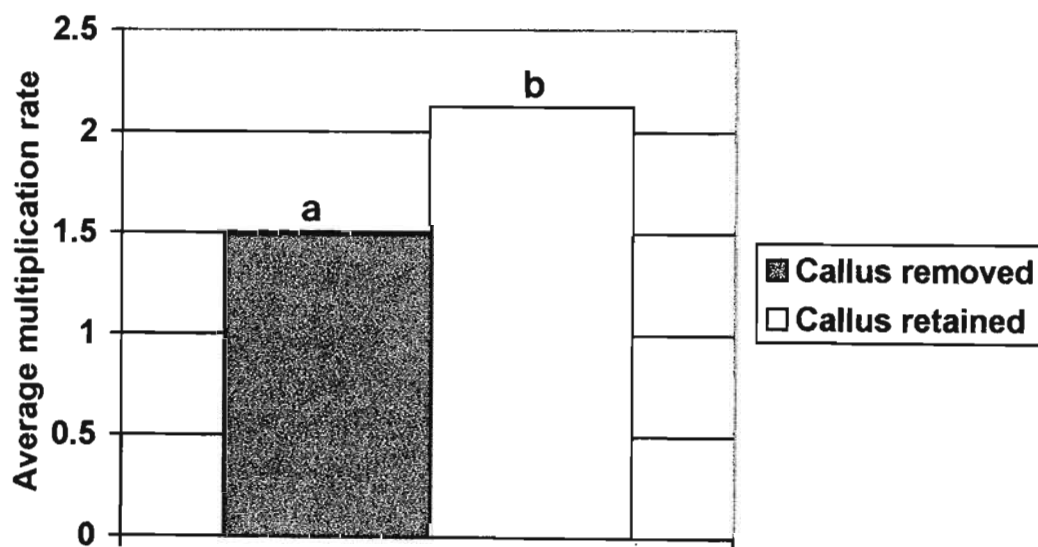


Fig. 27: The effect of callus removal from the bases of papaya *in vitro* on multiplication rate. LSD at 0.05 alpha level = 0.452 (ANOVA). Bars with the same letters are not significantly different at 0.05 alpha level

The function of the callus around the base of the plant is not known, but the above results (Fig. 27) show that callus is important for the multiplication of the papaya.

### **6.3.9 The effect of callus removal from the base of the plant on elongation of papaya**

The elongation of papaya *in vitro* was significantly greater after four weeks when the callus was not removed from the base of the plants than when it was removed (Table 19). Leaf colour and leaf abscission was also affected when the callus was removed, indicating that during elongation, it was beneficial to retain the callus around the base rather than removing it.

Table 19: The effect of callus removal from the base of the plant on overall appearance and elongation of papaya *in vitro*. ANOVA performed on the effect of callus on shoot growth showed significant differences at 5% level (indicated with \*)

<b>Treatment</b>	<b>Callus removed</b>	<b>Callus retained</b>
<b>Shoot growth (mm)</b>	14.7	22.4 *
<b>Leaf colour</b>	Yellow, veined leaves	Green
<b>Leaf drop (senescence)</b>	Yes	Little

### **6.3.10 Grafting of papaya**

#### **6.3.10.1 Grafting *in vitro***

The procedure described in 6.2.10.1 took about 10 minutes and was difficult to execute since the tissue culture material was very slippery and the grafts difficult to hold. Wedge grafting was also difficult because the material was too slippery and would not remain in the wedge. Although the sterile crepe bandage stuck very well, it was too large for the small diameter of stem (Fig. 26B).

*In vitro* grafting of papaya was impractical and time consuming using the methods described. It can not be recommended as a viable commercial procedure for propagation.

### 6.3.10.2 Grafting *ex vitro*

Although the *ex vitro* technique was also time consuming, it was a little easier since the rootstock seedling was firm and had a base. The unrooted *in vitro* material used for the scion had to be the same thickness as the seedling rootstock and was difficult to hold, as this too was slippery. It was also important to select green, healthy plants. The wedge grafts were more practical because the *in vitro* and *ex vitro* material slipped into each other without much need for support, while the slant graft needed to be held in place using one hand while wrapping the grafting tape with the other.

The small plastic pegs used to fasten the tape and the graft may have been a little too tight for the soft *in vitro* tissue, thus causing tissue damage (Fig 28A).

Table 20: Grafting of papaya using two types of grafting techniques and various grafting tape showing successful growth rates

<b>Tape used</b>	<b>Slant graft (%)</b>	<b>Wedge graft (%)</b>
Masking tape	0	0
Parafilm™	20	10
Grafting tape	40	40

The grafting tape was more effective than the Parafilm™ (Fig. 28B) and masking tape. Whether the slant (Fig. 28C) or the wedge grafting technique (Fig. 28D) was used did not make a difference to the overall percentage "take"(Table 20).

### 6.3.11 The effect of modified lids with cotton-wool plugs on leaf senescence

It is important to note that when the plantlets are pulsed with IBA solution, the leaves should not be submerged and that a period of two hours was too long. Also, only small volumes of plants must be handled at one time. In the present experiments the leaves absorbed too much water and became vitrified. No clear rooting result was achieved in this trial.

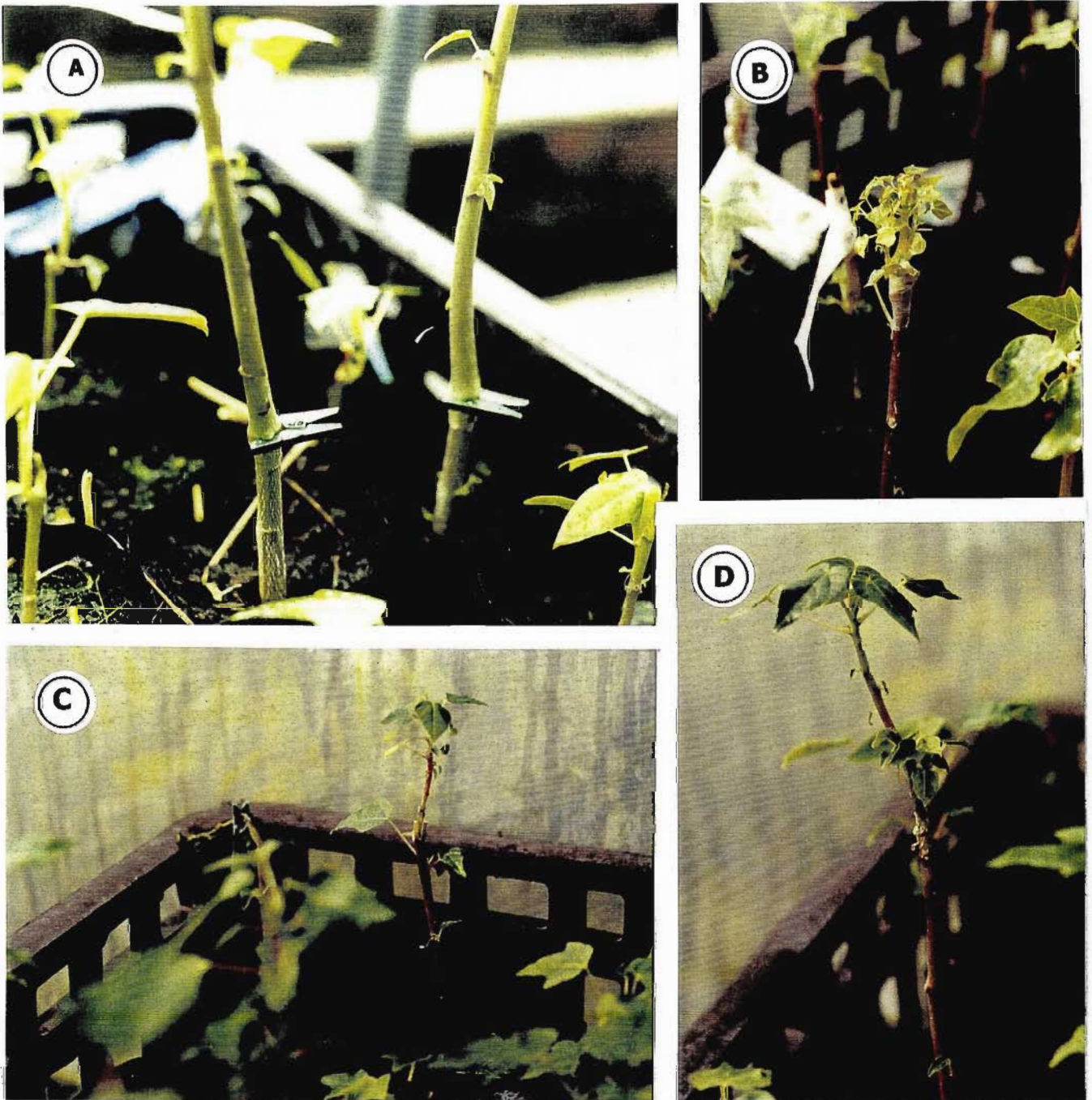


Fig.28A: *In vitro* plantlet grafted to *ex vitro* seedling rootstocks of papaya showing the the pegs used to clamp the graft; B: *In vitro* plantlet grafted to seedling rootstocks of papaya showing parafilm used to fuse the graft; C: Papaya *in vitro* material wedge grafted onto an *ex vitro* seedling rootstock; D: Papaya *in vitro* material slant grafted onto an *ex vitro* seedling rootstock

Cotton-wool plugs reduced the amount of senescence (Fig. 29) and in a separate experiment, the plugs were removed and the cultures kept in the growth room for a preliminary acclimatization period. Since no sucrose was added to the medium, the cultures did not become contaminated when the cotton-wool plugs (Fig. 30) on the lids of the vessels were removed. However, most of the leaves had fallen off and were being replaced by new leaves.

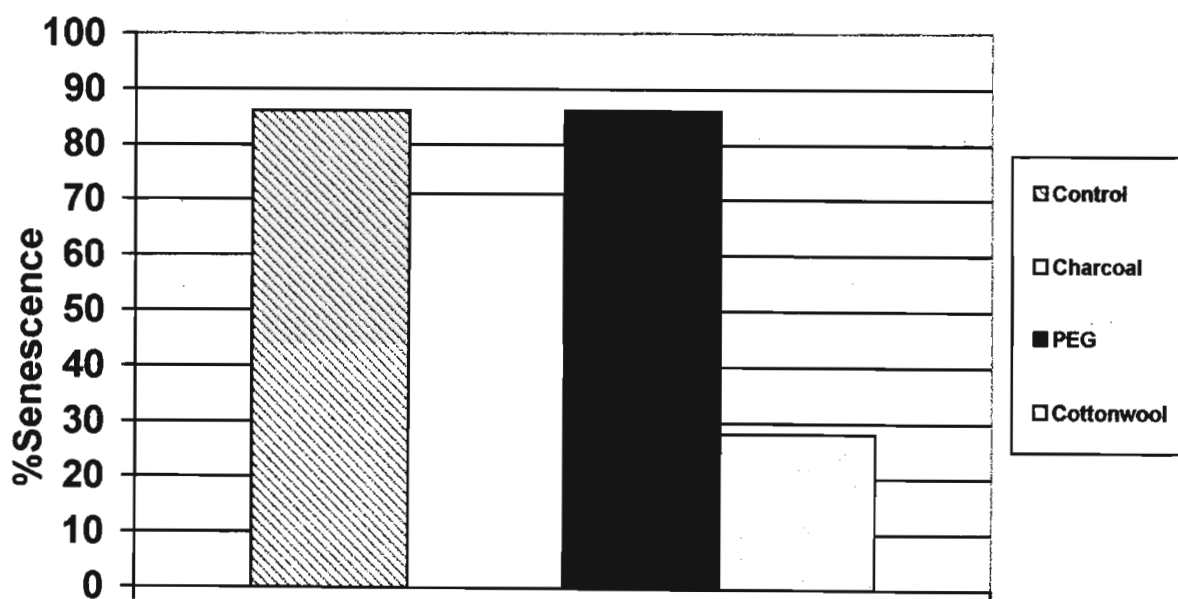


Fig. 29: The effect of various treatments on percentage senescence on papaya cultures *in vitro*



Fig. 30: Papaya plantlets grown on vermiculite medium with modified cotton-wool plug lids

#### 6.4 Discussion and Conclusions

With the commercial potential of *Carica papaya* var. Sunrise Solo in South Africa, a tissue culture protocol for the mass propagation thereof is required. This would enable production of a uniform, known, superior fruit. Although vegetative propagation by cuttings has been done with some success, when limited material is available, micropropagation through tissue culture allows for rapid propagation of many plants using limited material in a shorter period of time. Optimization of current papaya tissue culture techniques and protocols are required for a more efficient operating system. Contributing factors influencing multiplication and rooting of papaya such as sucrose

concentration, activated charcoal, gelling agents and cutting techniques were tested for their respective roles in overall *in vitro* papaya growth and rooting.

Sucrose played a significant role in the overall appearance and multiplication rate of papaya at a concentration of 50 g l<sup>-1</sup> giving the best result. Plants grown in the absence of sucrose or at lower concentrations of sucrose were weak and spindly compared to the plants grown on higher sucrose concentrations.

How the laminar flow bench operator handles the plant and how the plant is cut is an important factor in overall multiplication and growth of the plants *in vitro*. Cutting and removing the callus from the base of the plantlets reduced multiplication rates and elongation significantly. It was therefore important to not remove all callus from the bases of the plants. The role of the callus is not fully understood, but it is possible that some plantlets are formed from the callus clumps itself.

Activated charcoal had varying effects on the overall growth and rooting of papaya. The type of gelling agent used in combination with activated charcoal also influenced the response of activated charcoal on papaya *in vitro*. Although the best multiplication rate was achieved with plantlets supported on a medium solidified with Gelrite alone, the plants were vitrified and could not be used for a second subculture. Adding 3 g l<sup>-1</sup> activated charcoal to Gelrite assisted in obtaining non-vitrified plants. However, multiplication rate was reduced when activated charcoal was added to Gelrite compared to using Gelrite alone. This was also found when a combination of agar and Gelrite was used. No vitrification occurred with agar as gelling agent, but the multiplication rate was lower than the Gelrite treatment. Besides having an effect on multiplication rate, activated charcoal was found to be beneficial to the overall growth of plantlets. Leaves appeared greener, more mature, and an improvement on leaf senescence was observed with the addition of activated charcoal to the plant growth medium.

Another problem experienced with papaya micropropagation was obtaining a good rooting percentage and well-developed roots. Gelling agents play a role in overall rooting percentage and type of root formed *in vitro*. Lower concentrations of Gelrite, for example, were found to have a positive effect on rooting percentage. Lower

concentrations of agar also had a positive effect on overall growth and appearance of papaya at the rooting stage. It is possible that lower concentrations of gelling agents allowed for better aeration in the medium, but had the disadvantage that not enough support was given for the plantlets to remain upright in the vessels.

Papayas are very sensitive to plant growth regulators added to the medium. Toxicity effects with cytokinins have been reported (DREW, 1988), while high auxin concentrations are also known to have a negative effect on the roots. Plantlets exposed to IBA for only two days, then transferred to a medium free of plant growth regulators, showed a significantly higher rooting percentage than those plantlets left on the IBA medium for four weeks. The number and length of roots formed were also reduced. Activated charcoal is known to bind to certain compounds thus allowing for a "slow release" effect. The addition of activated charcoal to the medium containing IBA did not have a positive effect on overall rooting percentage, but did have an effect on the type of root formed. Activated charcoal has been reported to adsorb inhibitory compounds such as phenolics (WEATHERHEAD *et al.*, 1978) which may have had an effect on the type of root formed *in vitro*. The roots formed in the absence of activated charcoal were callused and tended to fall off easily and apparently did not have any vascular connections to the shoot vascular tissues. Smooth white roots formed in the activated charcoal medium and were more firmly attached to the shoot. However, those plants treated with IBA for two days and grown in the absence of activated charcoal had more and longer roots. It is possible that activated charcoal adsorbed the IBA, thus preventing the IBA from becoming toxic to rooting.

Since the results indicated that lower concentrations of gelling agents with better aeration capacities were beneficial to rooting the papaya, vermiculite as an alternative supporting medium was tested. Vermiculite provided the support required while at the same time it also allowed for better aeration in the medium.

In addition, the two-step procedure of first inoculating the plants on an IBA medium, incubating the plants for two days in the dark, then transferring them to a plant growth regulator free medium for the remaining three and a half weeks in the light, was tedious and impractical. A one-step method of dipping the plantlets in an IBA solution for one

hour, then transferring it to the vermiculite medium was tested with positive results. Eighty percent rooting was achieved with good roots forming around the stem and base. The IBA concentration used was higher than previously used in the two-step method. The experiment was repeated soaking the plantlets in the IBA solution for two hours. However, this cannot be recommended, as the plantlets tended to sink into the solution, which was absorbed by the leaves, and became vitrified. It was important to only submerge the bases of the plants.

Using cotton-wool plugs in small 1 cm diameter holes in the vessel lids did have an effect on reducing leaf abscission and overall senescence. The cotton-wool plugs could be easily removed and the plantlets gradually exposed to the outer environment while still *in vitro*. Although the acclimatization success was not tested, the method describes a pre-acclimatization step that may help to reduce losses in the greenhouse. The papaya is known to be very sensitive to changes in humidity at the acclimatization stage.

An alternative propagation method was tested by grafting an unrooted *in vitro* plant onto an established seedling in the greenhouse using wedge and slant grafts. The *in vitro* material proved to be very slippery and difficult to handle, although a limited degree of success was achieved.

It is possible, that with practice, the success rate of the technique applied above may improve, and could be a valuable tool for the propagation of papaya and other difficult-to-root plants. The plant must be able to overcome the hardening shock as well as being able to graft to a seedling. Following this trial, *ex vitro* grafts were made of superior tissue culture selections to cold tolerant rootstocks. This technique will improve the selection of the papaya for various regions, but still maintain the fruit quality required. At present, however, *in vitro* grafting is time consuming and impractical. A better technique will have to be developed for it to be commercially viable.

As most cultivars behave differently *in vitro* and require different growing conditions, the technique recommended may not apply to all papaya cultivars, but in general, the concepts used can be tested and evaluated for future varieties and hopefully contribute to a better, more efficient method of micropropagation.

## CHAPTER 7

### OVERALL CONCLUSIONS

The study deals with two economically and agriculturally important plantation crops: date palm (*Phoenix dactylifera* L.) and papaya (*Carica papaya* L.). Date palms are dioecious and because the sex of the palm is not known until it bears fruit four years after establishment, tissue culture plants of superior female cultivars have become the popular choice of establishment propagation material. Furthermore, diseases and pests have destroyed many existing plantations and the demand for planting material exceeds 5 million plants. Off-shoots are in short supply and may carry diseases. Due to the demand for tissue culture plants, laboratories and governments around the world are seeking the best protocol to rapidly multiply these plants.

Various techniques are available and some have been published, however, they are not without their problem areas and the processes still require optimization. Furthermore, there is much debate on which technique (direct and indirect somatic embryogenesis and organogenesis) is best used commercially. While somatic embryogenesis is an efficient technique in terms of time taken to produce a tissue culture plant, it carries the risk of somaclonal variation as it undergoes dedifferentiation to callus. Direct somatic embryogenesis uses continuous embryo cultures and thus carries less risk of variation. However, this study shows that growth variations did occur among plants produced using this technique. No variations thus far have been reported for plants produced using organogenesis.

A southern African survey was conducted for off-types on date palms produced by somatic embryogenesis. Leaf variegation, compact growth forms, stem bending, broader leaf structure and parthenocarpic and seedless fruit were among the abnormalities reported. Environmental, physiological and genetic factors all contribute to possible explanations for these variations in growth. Genetic mutations can only be verified using molecular technology. Although much research has been done to assist in variety identification, no technique has to date been developed to involve the entire genome to detect specific mutations. The study clearly showed that abnormalities do occur on

tissue cultured plants and it therefore lies in the hands of the tissue culture laboratory to ensure that sound techniques are practiced.

Initiating date palms is laborious and takes many months before callogenesis or shoot proliferation occurs. Optimization of this process is vital in achieving a more efficient propagation system. A double decontamination in 2,6 % then 1,3 % NaOCl was efficient at reducing contamination of explants. An explant size of 1 cm<sup>3</sup> was most responsive in somatic embryogenesis. TIBA had no effect on somatic embryogenesis at 25 mg l<sup>-1</sup>. It is possible that with the lack of another auxin applied, the anti-auxin effect did not occur, thus not initiating a visible response. NAA also had little effect on somatic embryogenesis. Earlier somatic embryogenesis was achieved using "nurse cultures". Substances secreted into the medium by the mature plantlet may have influenced the earlier dedifferentiation of the explant. Reducing the time spent *in vitro* at initiation is a major advantage.

In the second part of the study, the effect of various techniques, gelling agents and nutrient media on papaya micropropagation were investigated. Papaya, like the date palm, is in demand and planting material for selected varieties is in short supply. For uniformity of plantations and fruit, micropropagation provides a rapid means of propagation. Various problems still exist with the micropropagation of papaya. The focus of this study was therefore to provide an improved yet practical technique for papaya tissue culture.

During initiation, contamination may be reduced by presoaking explants in the antibiotic, Rifampicin. Other decontaminating agents such as Bronocide™ and combinations of fungicides and antibiotics would not be recommended as they had little effect on reducing contamination, and in some cases, phytotoxic effects on explants were observed at the concentrations used.

The study demonstrated that it was important to retain some of the callus during the multiplication and elongation process of the papaya. Gelrite was not a suitable gelling agent for multiplication because, although higher multiplication rates were achieved, the plantlets became vitrified. Combinations of Gelrite and agar or Gelrite and AC reduced vitrification. The growth and multiplication of papaya was optimal at 50 g l<sup>-1</sup> sucrose.

Activated charcoal was shown to play an important role in overall improved plant appearance and growth. It is possible that AC adsorbs excess plant growth regulators and other inhibitory substances in the medium, thus reducing the toxicity effects reported by DREW, 1988. The inclusion of 0.3 % AC to the medium for one subculture prior to root initiation will not only improve overall shoot development, but may also improve subsequent rooting percentage. Healthy, strong shoots are not only important for root initiation, but also have an effect on acclimatization (CHEE and POOL, 1988).

The use of vermiculite as a supporting medium with a one-hour pulse of 5mg l<sup>-1</sup> IBA on plantlets for rooting was an efficient one-step method to obtain good roots. Roots formed on agar and Gelrite were inferior when compared to vermiculite used *in vitro* that provided a porous aerated medium for good root formation. Although only 80% rooting in vermiculite medium was reported in this study, the method eliminates tedious reculturing to fresh medium free of IBA after a three day exposure. Another advantages to using vermiculite was that root hairs are formed and there was no need to rinse off any adhering substances prior to transferring plantlets to potting medium for acclimatization. The use of cotton-wool plugs on lids of the vessels reduced leaf senescence and could be easily removed to also assist in pre-acclimatizing the plants for the greenhouse.

*In vitro* grafting using stericrepe proved impractical, while grafting *in vitro* unrooted papaya plants onto *ex vitro* seedlings was more successful, using wedge and slant grafts. Grafts sealed with pegs and Parafilm™ were less effective. Although this method was not commercially viable, it may prompt initiatives in grafting cold tolerant or disease resistant rootstocks with superior tissue culture material.

To conclude, this study showed a number of improvements over previously published techniques on papaya micropropagation (DREW, 1992; DREW and SMITH, 1986; RAJEEVAN and PANDEY, 1986). Activated charcoal improved overall growth at multiplication, vermiculite medium improved root growth and the rooting procedure was made faster and more cost-effective, thus fulfilling the aims of the study.

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