IN VITRO CULTURE OF AVOCADO: A MODEL SYSTEM FOR STUDYING THE BIOCHEMISTRY OF FRUIT GROWTH

By

Dudley Ian Mitchell

Submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCES IN AGRICULTURE

in

Horticultural Science

School of Agricultural Sciences and Agribusiness

Faculty of Science and Agriculture

University of Natal

Pietermaritzburg

January 2000

ABSTRACT

The avocado (*Persea americana* Mill.) is considered by many to be a horticultural problem. Cultivars in use today have several drawbacks associated with yield, disease susceptibility and fruit quality. Breeding programs to counter these disadvantages have been met with limited success due mainly to the crop's heterozygosity, outbreeding nature and long juvenile period. Furthermore, genetic information regarding current commercial scions and rootstocks is limited and crosses are made based on parental phenotypic characteristics which are not always additive. It is proposed that the development of a protoplast-to-plant system for avocado would not only provide a means for plant breeders to overcome these problems, but would also present researchers with a useful tool for studying biochemical and physiological mechanisms operating within the plant. An investigation into the development of an *in vitro* system for use in metabolic studies was carried out. This technology was then used as a model system for studies into the metabolic control of cell growth.

An attempt was made at developing a protoplast system from the mesocarp tissue of 'Hass' avocado. It was found that the purity and activity of the cellulase preparation in the protoplast isolation medium was critical. Failure to generate a protoplast system from mesocarp tissue prompted an investigation into the development of cell cultures. Mesocarp, seed and embryo tissue was subjected to various treatments in an attempt to induce callus for use as a source material for cell cultures. Callus derived from nucellar tissue of 'Hass' avocado seed at high concentrations of α-naphthalene acetic acid (NAA)(5 mgL^{-1}) and isopentenyladenine (iP)(5 mgL^{-1}) in Murashige and Skoog media (MS) proved to be the most amenable to subculture into liquid medium. Cell suspensions initiated from this callus grew fastest in MS media supplemented with NAA (5 mgL^{-1}) and iP (1 mgL^{-1}). These cell suspensions were maintained through subculture and were selected for use in metabolic studies.

Cytokinin-dependent cell cultures from avocado seed callus were used to study the involvement of isoprenoid products in cell division. Addition of mevastatin, a competitive inhibitor of the key enzyme in the isoprenoid pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), caused a reduction in cell growth at low concentrations (0.01 μM, 0.1 μM and 1 μM) and cessation of growth at higher concentrations (10 μM and 40 μM). Co-treatment with the isoprenoid compounds mevalonic acid lactone (MVL)(6 mM) and farnesyl diphosphate (FDP)(10 μM) completely reversed the effects of mevastatin at the 1 μM and 40 μM levels. The addition of stigmasterol (10 μM) to cell cultures treated with mevastatin (1 μM and 40 μM) resulted in a slight positive growth response indicating partial alleviation of inhibition. However, the response was not significantly different from the control suggesting that sterols played a minor role in cell division. It was concluded that isoprenoid-derived products played a critical role in the regulation of the cell cycle. Furthermore, it was suggested that mevastatin-induced HMGR inhibition gave rise to a response, most likely ABA-mediated, that acted antagonistically to regulatory mechanisms controlled, in part, by isoprenoid compounds.

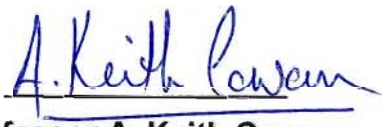
DECLARATION

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

Signed: 

Dudley Ian Mitchell

I hereby certify that this statement is correct.

Signed: 

Professor A. Keith Cowan

Supervisor

Horticultural Science

School of Agricultural Sciences and Agribusiness

Faculty of Science and Agriculture

University of Natal

Pietermaritzburg

January 2000

ACKNOWLEDGEMENTS

The author would like to express his sincere gratitude to the following people and organisations:

1. Professor A. K. Cowan for his patience, encouragement and motivation over the course of this research.
2. The Foundation for Research and Development (GUN 2034569) and the University of Natal for financial support.
3. Teri Dennison, Paul Hildyard and Lowie de Klerk for their willing administrative assistance and their help in acquiring chemicals, facilities and services.
4. Vehitha Beharee, Ken Cradock, Kerry Hughes and the staff of Microbiology for their guidance and use of their facilities.
5. Nicky Greeff for her patience, love, support and continual encouragement over the years.
6. Mum and Dad for your love, support, encouragement and guidance in life.

Mum

"The soul would have no rainbow had the eyes no tears"

To thee
Science appears but, what in truth she is,
Not as our glory and our absolute boast,
But as a succedeneum, and a prop
To our infirmity

William Wordsworth, 1850, *The Prelude*

TABLE OF CONTENTS

ABSTRACT (i)
DECLARATION (iii)
ACKNOWLEDGEMENTS (iv)
LIST OF ABBREVIATIONS (viii)
1. GENERAL INTRODUCTION 1
1.1 THE AVOCADO – A HORTICULTURAL PROBLEM 2
1.2 THE *IN VITRO* CULTURE OF AVOCADO 4
1.2.1 The current state of avocado tissue culture 6
1.2.2 Potential areas of application of tissue culture techniques in avocado production 9
1.2.2.1 Propagation 10
1.2.2.2 Breeding 11
1.2.2.3 Research 17
1.3 EPILOGUE 20
1.4 OBJECTIVES 20

2. MATERIALS AND METHODS 21
2.1 CHEMICALS 21
2.1.1 Enzymes and fine chemicals 21
2.1.2 Sugars 21
2.1.3 Prepared media 22
2.1.4 General chemicals 22
2.2 PREPARATION OF MEDIA 22
2.2.1 Protoplast isolation 22
2.2.1.1 Chapman, Power and Wilson (CPW) salts 22
2.2.1.2 Citrus protoplast isolation medium (CPIM) 23
2.2.1.3 Carrot protoplast isolation medium (CtPIM) 24
2.2.1.4 Sorghum protoplast isolation medium (SPIM) 24
2.2.1.5 Avocado protoplast isolation medium (APIM) 24
2.2.2 Protoplast culture 25
2.2.3 Callus induction and cell suspension culture 26
2.3 PREPARATION OF GROWTH REGULATORS, ISOPRENOIDS AND STAINS 28
2.4 PLANT MATERIAL 29
2.5 PROTOPLAST ISOLATION FROM THE MESOCARP OF 'HASS' AVOCADO FRUIT 29
2.6 CALLUS INDUCTION 30
2.7 INITIATION AND MAINTENANCE OF CELL SUSPENSION CULTURES 32
2.8 CULTURE CONDITIONS FOR PROTOPLASTS, CALLUS AND CELL SUSPENSION CULTURES 33
2.9 PROTOPLAST CULTURE 33
2.10 ISOLATION OF PROTOPLASTS FROM CELL SUSPENSIONS OF 'HASS' AVOCADO 34
2.11 TREATMENT OF CALLUS AND CELL CULTURES 35
2.11.1 Callus subculture 35
2.11.2 Callus manipulations 35
2.11.3 Cell suspension subculture 37
2.11.4 Population dynamics of 'Hass' avocado cell suspensions 37
2.11.5 Reversal of mevastatin-induced cell cycle arrest by the addition of mevalonic acid lactone, farnesyl diphosphate and stigmasterol 38

2.12 MICROSCOPY 38
2.12.1 Cell counting with a hemacytometer 38
2.12.2 Cell counting with a Sedgewick Rafter counting chamber 39
2.12.3 Light microscopy 39
2.12.4 Fluorescence microscopy 39
2.12.4.1 Protoplast viability 39
2.12.4.2 Viability of cultured cells 40
2.12.5 Scanning electron microscopy of protoplasts 40
2.13 DATA ANALYSIS 41
2.13.1 Digital image acquisition and modification 41
2.13.2 Callus evaluation 41
2.13.3 Statistical analysis 41

3. DEVELOPMENT OF AN *IN VITRO* CULTURE SYSTEM FROM AVOCADO FRUIT 43
3.1 INTRODUCTION 43
3.2 RESULTS 46
3.2.1 Protoplast isolation from mesocarp tissue 46
3.2.2 Callus induction 52
3.2.2.1 Mesocarp explants 52
3.2.2.2 Seed explants 55
3.2.2.3 Embryo explants 60
3.2.3 Initiation and maintenance of cell suspension cultures 62
3.2.4 Culture of mesocarp protoplasts 68
3.2.5 Isolation of protoplasts from cell suspensions 68
3.3 SUMMARY 71

4. INVESTIGATIONS INTO THE CONTRIBUTION OF THE ISOPRENOID PATHWAY TO GROWTH OF CELL SUSPENSION CULTURES 74
4.1 INTRODUCTION 74
4.2 RESULTS 77
4.2.1 Investigations into population dynamics of 'Hass' avocado cell suspensions 77
4.2.2 Reversal of mevastatin-induced inhibition of cell growth 80
4.3 SUMMARY 84

5. GENERAL DISCUSSION AND CONCLUSION 86
5.1 GENERAL DISCUSSION 86
5.1.1 Development of an *in vitro* culture system from avocado fruit 86
5.1.2 Investigations into the contribution of the isoprenoid pathway to growth of cell suspension cultures 93
5.2 CONCLUSIONS AND FUTURE PROSPECTS 100

LITERATURE CITED 101

LIST OF ABBREVIATIONS

BA, 6-benzylaminopurine

BSA, bovine serum albumin

CDK, cyclin-dependent protein kinase

DMSO, dimethylsulfoxide

DTT, 1,4-ditiothreitol

FB28, fluorescent brightener 28

FDA, fluorescein diacetate

FDP, farnesyl diphosphate

Ftase, farnesyl transferase

GCP, guard cell protoplasts

GGPP, geranylgeranyl diphosphate

GGTase, geranylgeranyl transferase

GPP, geranyl diphosphate

HDL, high density lipoproteins

HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase

IBA, indole butyric acid

iP, 6-(γ,γ-dimethylallylamino)-purine

Kn, 6-furfurylaminopurine

LDL, low density lipoproteins

MS, Murashige-Skoog basal salt media

MSB, Murashige-Skoog basal salt media with B5 vitamins

MVA, mevalonic acid

MVL, mevalonic acid lactone

NAA, α-naphthalene acetic acid

CHAPTER 1

GENERAL INTRODUCTION

The avocado (*Persea americana* Mill.) is of tropical to subtropical origin and is cultivated in diverse environments ranging from the humid tropics to semi-arid, Mediterranean-type areas up to latitudes of 43° (Whiley, 1994; Wolstenholme and Whiley, 1995; Gaillard and Godefroy, 1995). Botanically, the avocado fruit is described as a berry consisting of a single seed surrounded by a thick, fleshy mesocarp and enclosed by a smooth or 'warty' skin (Whiley and Schaffer, 1994; Gaillard and Godefroy, 1995). It is pyriform or glabose in shape and can have a skin colour ranging from yellow-green to dark purple at maturity (Whiley and Schaffer, 1994; Gaillard and Godefroy, 1995). Taxonomically, the cultivated avocado belongs to the aromatic family *Lauraceae* and the valid genus *Persea* (Scora and Bergh, 1990). Three horticultural races are recognized within the species *P. americana* and are granted varietal status according to their presumed centres of origin. Although controversy still exists concerning the particular origins, species and varietal names, the three races are generally accepted as *P. americana* var. *americana* (West Indian race), *P. americana* var. *drymyfolia* (Mexican race) and *P. americana* var. *guatamalensis* (Guatamalan race)(Whiley and Schaffer, 1994; Bergh and Ellstrand, 1986; Storey *et al.*, 1986). These three races hybridize freely, and this outbreeding nature, coupled with thousands of years of climatic adaptation and human selection and cultivation, has resulted in many inter-racial hybrids, some of which have become the main present day commercial cultivars (Storey *et al.*, 1986; Scora and Bergh, 1990; Nakasone and Paull, 1998).

While not attaining the same status as *Citrus* (citrus), *Musa* (bananas) and deciduous fruit in terms of world consumption and production, the avocado is attracting increasing attention from health orientated first world countries because of its nutritive value and apparent benefits in cosmetic care. This obviously bodes well for the future of avocado industries the world over but especially in developing countries, like South Africa. From 1993 to 1995,

South Africa produced 2% of the world avocado crop of which the main cultivars were the Guatamalan x Mexican hybrid clones, 'Hass' and 'Fuerte' (van Zyl and Ferreira, 1995). A worrying statistic, for the South African industry, in terms of competition, and the world industry, in terms of stability, is that the large majority of producers are reliant on these two cultivars (van Zyl and Ferreira, 1995; Nakasone and Paull, 1998) and the implications of over production or a fatal disease outbreak, for example, are obvious. In addition, lack of available material for in depth physiological and biochemical experimentation due to seasonality, reduced technology transfer between producing countries because of distances and phytosanitary controls, and the relatively recent history of commercial domestication of the species, have hindered progress in production, breeding and research technology. This has resulted in an industry that is struggling to hold its place on the world scene in spite of its obvious potential.

1.1 THE AVOCADO – A HORTICULTURAL PROBLEM

Although the avocado has a long history of use (archeological evidence suggests that the Mexican avocado has been used as a food source for ±10000 years (Williams, 1976)) and has undoubtedly been improved by both artificial and natural selection, there is still considerable potential for improvement of current cultivars (Bergh, 1987). Problems associated with the cultivars in use today include:

- low yield of available scion/rootstock combinations,
- alternate bearing,
- a relatively short marketing season,
- disease susceptibility of scions and rootstocks,
- relatively poor keeping quality,
- post harvest physiological disorders (Knight and Winters, 1971; Slor and Spodheim, 1972).

To address these problems, many national industries have established breeding programmes aimed at selecting superior lines suited to the particular

needs of that specific area of cultivation / production (Bijzet *et al.*, 1995; Bergh, 1987; Lahav *et al.*, 1995). The avocado, however, is characterized by several traits that have impeded conventional breeding techniques. These include, high heterozygosity, a flowering mechanism suited to outbreeding and a long juvenile phase before fruit production (Lavi *et al.*, 1991; Lavi *et al.*, 1992; Nakasone and Paull, 1998). Together, these factors have resulted in limited success in the development of improved scion and rootstock cultivars and growers (especially in South Africa) are still largely reliant on the selections, 'Hass' and 'Fuerte' which arose as chance seedlings and of which the genetics are still obscured (Bergh, 1976; Davis *et al.*, 1998). In addition, until recently, the genetic information available for most current commercial scion and rootstock cultivars has been limited and crosses have been made based on parental phenotypic characteristics under the assumption that the genetic traits were additive (Lavi *et al.*, 1993). In practice, however, the performance of offspring from most crosses is unpredictable and studies have shown that non-additive genetic variance is a significant component of total genetic variance (Lavi *et al.*, 1991; Lavi *et al.*, 1993).

The above mentioned characteristics highlight the need for the development of rapid breeding methods where selection procedures can be applied to genetically manipulated tissue cultures for the production of novel genotypes with known parentage. A protoplast-to-plant tissue culture system would be integral to such a system and would act as an intermediary between the associated areas of molecular biology, breeding and genetics (Smith and Drew, 1990). While the existence of a protoplast-to-plant system for *P. americana* would provide the required technology for improved breeding opportunities, it would also present a useful tool for other research applications related to biochemical and physiological mechanisms within the species.

1.2 THE *IN VITRO* CULTURE OF AVOCADO

Since Haberlandt (1902) first published the idea of totipotency in plant cells, plant tissue culture has evolved tremendously and now encompasses a wide range of technologies which have become integral to both the commercial plant production industries as well as to particular crop research programmes (Smith, 1994). With respect to commercial plant production, plant tissue culture offers a convenient and beneficial means of mass propagating economically valuable selections and new crop introductions with additional improvements in production timing, product uniformity, efficiency, availability of clean, virus-free material and flexibility in response to market demands (Smith, 1994). In research, plant tissue culture has become an integral part of molecular approaches to plant improvement, acting as a means whereby, advances made in molecular biology in the areas of gene isolation and modification, can be transferred to plant cells and the resulting selections of superior genotypes can be identified (Smith and Drew, 1990; Smith, 1994). In addition, plant tissue culture has found an increasing application in research as a vehicle for in-depth physiological and biochemical investigations (Smith, 1994). Fig. 1.1 illustrates the role tissue culture plays in advanced plant breeding and research applications and includes the spectrum of present day *in vitro* culture techniques against which the potential applications to avocado will be considered.

While protoplast-to-plant systems have been developed for many herbaceous plant species (Ochatt and Patat-Ochatt, 1995), woody species were generally regarded as being recalcitrant (Ochatt and Power, 1992; Ochatt *et al.*, 1992) except *Citrus*, in which a protoplast-to-plant system was developed as early as 1975 (Vardi *et al.*,1975) and commercial cultivar releases from somatic hybridization are due soon (P. Bird, pers. comm.[1]). The late 1980's saw increased interest and success in the development of protoplast-to-plant systems for several woody species and fruit trees, demonstrating that such plants were not beyond the scope of biotechnological breeding approaches (Ochatt *et al.*, 1992). Besides *Citrus*, however, research into such systems for

[1] P. Bird, 1999, ARC, Institute for Tropical and Subtropical Crops, Addo, ZA

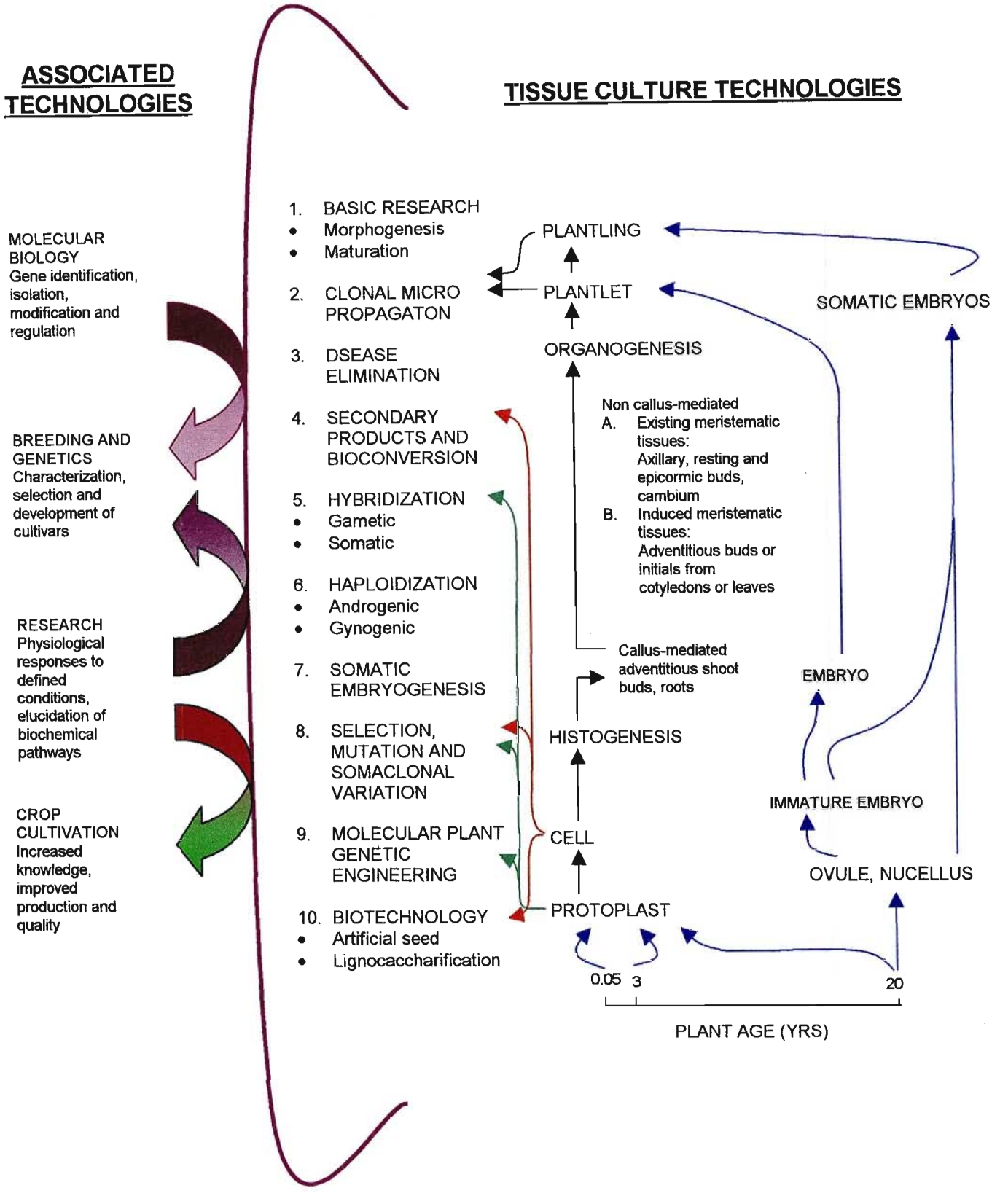


Figure 1.1

Actual and potential applications of *in vitro* technology to *P. americana*. The right hand side presents the relationships between the various technologies available while the left hand side illustrates the use of tissue culture as an intermediate technology in crop improvement (after Bornman, 1987; Smith and Drew, 1990)

other tropical and subtropical species has been limited and, while plants of important temperate, fresh fruit crops such as apple (*Malus* x *domesticus* Borkh)(Patat-Ochatt *et al.*, 1988) and pear (*Pyrus communis*)(Ochatt and Power, 1988) have been regenerated from isolated protoplasts, only *Coffea canephora* (Schopke *et al.*, 1987) and *Coffea arabica* (Yasuda *et al.*, 1986) have been added to the list of tropical and subtropical fruit crops. The problems associated with the cultivated avocado and inherent evolutionary adaptation of the crop and its potentially bright economic future, provide a compelling case for the development of a full protoplast-to-plant system with all the associated technologies for this species.

1.2.1 The current state of avocado tissue culture

The technologies researched in the development of *in vitro* techniques for avocado are presented in Table 1.1.

While studies into the tissue culture of avocado started over 40 years ago, the development of *in vitro* culture systems is still regarded as being in its infancy (Pliego-Alfaro and Bergh, 1992). Whole plants are purported to have been regenerated from embryos and shoot tips (Pliego-Alfaro and Murashige, 1987; Gonzalez-Rosas *et al.*, 1985, Gonzalez-Rosas *et al.*, 1990; Nel *et al.*, 1982) although the rooting of embryos *in vitro* was considered to be too slow and variable to be of practical value (Skene and Barlass, 1983). In addition, regeneration of whole plants from shoot tips was achieved only in relatives of avocado - *Persea schiediana* Ness. (Gonzalez-Rosas *et al.*, 1985) and *Persea indica* (Nel *et al.*, 1982) which were considered to be of some value in rootstock breeding programs. Recently, however, it has been shown that complete plants can be regenerated from embryogenic axes (Mohamed-Yasseen, 1992) or transversely dissected embryogenic shoots (Ahmed *et al.*, 1998). In both cases, shoots are induced on a basal medium from embryos and then transferred to a shoot multiplication medium. The multiple adventitious shoots which are formed are then subcultured onto an induction medium for root formation. In accordance with Skene and Barlass (1983), Mohamed-Yasseen (1992) reported that the percentage of rooted shoots was

Table 1.1
Avocado tissue culture

Technology	Explant	Type of morphogenesis	Reference
Embryo culture	Mature embryo	Germination	Pliego-Alfaro and Murashige (1987); Gonzalez-Rosas *et al.* (1990)
	Immature embryo	Shoot formation	Skene and Barlass (1985)
		Somatic embryos	Mooney and Van Staden (1987); Piego-Alfaro and Murashige (1988)
Callus initiation	Shoot tips and axillary buds	Callus	Desjardins (1958); Schroeder (1973)
	Stem	Callus	Van Lelyveld (1984)
	Leaf	Callus	Young (1983)
	Flower	Callus	Schroeder (1975)
	Fruit mesocarp	Callus	Schroeder (1955; 1956; 1961; 1963; 1967; 1968; 1971); Schroeder and Kay (1961); Schroeder *et al.* (1962) Gazit and Blumenfeld (1970; 1971); Blumenfeld and Gazit (1971)
	Cotyledon	Callus	Kay and Schroeder (1963); Schroeder (1968; 1977); Gazit and Blumenfeld (1970; 1971)
	Protoplast	Callus	Blickle *et al.* (1986)
Shoot induction (organogenesis)	Immature embryo	Shoots	Skene and Barlass (1983)
	Shoot tips and axillary buds	Shoots	Schroeder (1976; 1979; 1980); Solorzano-Vega (1989); Young (1983); Gonzalez-Rosas and Salazar-Garcia (1984)
Whole plant regeneration	Mature embryo	Complete plant	Pliego-Alfaro and Murashige (1987); Gonzalez-Rosas *et al.* (1990)
	Embryonic axes	Complete plant	Mohamed-Yasseen *et al.* (1992); Mohamed-Yassen (1992); Ahmed *et al.* (1998)
	Shoot tips and axillary buds	Complete plant	Gonzalez-Rosas *et al.* (1985)*; Nel *et al.* (1982)**
Androgenesis	Pollen	Germination	Sahar and Spiegel-Roy (1984)
Somatic embryogenesis	Immature embryo	Somatic embryos	Mooney and Van Staden (1987); Piego-Alfaro and Murashige (1988)
Cell suspensions	Stem		Van Lelyveld (1984)
Protoplast isolation and culture	Callus	Protoplast/callus	Blickle *et al.* (1986)
	Fruit mesocarp	Protoplast	Percival *et al.* (1991)

*Plant material taken from *Persea schiedeana*

**Plant material taken from *Persea indica*

low whilst the results of Ahmed *et al.* (1998) showed that only 60% of explants formed adventitious roots. In contrast, Pliego-Alfaro (1988), while developing a rooting bioassay for avocado using juvenile phase stem cuttings from *in vitro* germinated seed, reported that almost every shoot rooted, in a similar, two-step procedure. Clearly, a large amount of variability exists in this area of avocado tissue culture and much research needs to be done to develop an efficient regeneration system.

Direct organogenesis from organized tissue, as in the above examples, however, is not the only regeneration mechanism available and is, in fact, rather limited in terms of transformation for breeding purposes and use in physiological studies. The efficient *de novo* regeneration of plants from cell and tissue cultures is integral to the application of most genetic approaches to crop improvement (Litz and Gray, 1992). Many transformation procedures such as *Agrobacterium*-mediated and particle gun manipulations rely on there being a proven protocol for plantlet formation as do the protoplast technologies of electroporation, cybrid production and somatic hybridization. While adventive organogenesis is frequently used as a regeneration pathway, it is often labour intensive and subject to a large amount of variation (Lorz *et al.*, 1988). Adventive embryogenesis, on the other hand, is an attractive alternative, offering complete microplants in potentially synchronous cultures initiated from single cells. Somatic embryogenesis is the initiation and development of an embryo from cells that are not the product of gametic fusion (i.e. asexual or somatic)(Tisserat *et al.*, 1980). In avocado, somatic embryogenesis has been reported only twice (Mooney and Van Staden, 1987; Pliego-Alfaro and Murashige, 1988) and in both instances, although microscopic evidence proved the existence of such structures, normal regeneration was restricted to a very limited percentage. Both adventive organogenesis and embryogenesis involve the production of callus from which organized growth in the form of shoots or roots (organogenesis), or a bipolar structure ('embryo') with a closed vascular system (embryogenesis) arises. The callus stage is fundamental to tissue culture. From this disorganized growth, many biotechnologies have been developed and, in itself, it represents an extremely useful tool for in depth studies on plant growth,

development and responses. The derivation of callus from explants is well documented for avocado. Almost every organ of the tree was subjected to experimentation on callus induction by Schroeder (1955; 1956; 1957; 1961; 1963; 1967; 1968; 1971; 1973; 1975; 1977) and, while this contributed significantly to the basic research knowledge of avocado tissue culture, no exceptionally noteworthy research was conducted with this tissue. The versatility of callus tissue has made it an extremely useful starting point for the initiation of cell suspension cultures and as a source material for protoplast isolation. With regard to avocado, very little research has been carried out in the areas of cell culture and protoplast isolation and culture. Van Lelyveld (1984) published a short report on avocado physiological disorders which contained a brief reference to cell suspensions, while Blickle *et al.* (1986) presented a poster at the International Congress of Plant Tissue and Cell Culture outlining the conditions for the isolation of protoplasts from callus cultures, but no publication has been forthcoming. The only substantial report on the isolation and use of avocado protoplasts was published by Percival *et al.* (1991) who developed a model system for ripening studies using protoplasts isolated from fruit mesocarp tissue.

If the technologies developed for avocado, (Table 1), are compared with those presented in Fig. 1.1, it can be concluded that there is a void in information regarding avocado tissue culture. Considering the limitations facing avocado breeding and cultivation, as well as the problems faced by particular growers in fruit production and marketing, there seems to be vast potential for the research into, and the application of, tissue culture techniques.

1.2.2 Potential areas of application of tissue culture techniques in avocado cultivation

There are basically three areas of avocado production in which tissue culture can be used to improve or compliment current practices in terms of speed, efficiency and fundamental crop behaviour. These areas include propagation, breeding and crop research.

1.2.2.1 Propagation

Plant tissue culture was first introduced as a rapid and reliable method to propagate plants asexually (Skirvin *et al.*, 1994). As such, it holds tremendous potential for the cultivation of *P. americana*. The avocado industry of today is essentially based on seedling rootstocks (Ben-Ya'acov and Michelson, 1995). Current propagation practices (in South Africa since 1987) involve the use of clonal material but the process is difficult and costly especially since an avocado seedling from first graft to planting-out spends approximately one year in the nursery (Ben-Ya'acov and Michelson, 1995; Smith and Drew, 1990). In addition, cloning material is not always uniform, and mixed results, in terms of yield and quality have been reported (Coffey, 1992 (cited by Ben-Ya'acov and Michelson, 1995)). According to Whiley *et al.* (1990), plants grafted on clonal rootstocks are more difficult and slower to establish than trees grafted onto seedling rootstocks. In spite of these problems, it is expected that clonal propagation of cultivated avocado will continue until such time as a better, more efficient, more uniform technology is developed.

With respect to the above, Smith and Drew (1990) state that the micropropagation of rootstocks and compatible scions would be of immense benefit to the industry. They go on to say that the biggest single gains in productivity of subtropical tree crops can be made via genetic uniformity of planting material. The efficient, regular regeneration of avocado plants from organized structures *in vitro* (micropropagation) has still not been achieved although there are indications that this possibility is not far off (Pliego-Alfaro, 1988; Mohamed-Yasseen, 1992; Ahmed *et al.*,1998). As promising as this might seem, the use of clonal propagation through tissue culture carries with it a risk of non-uniformity as big or bigger than the use of clonal propagation through the grafting of cuttings. Indeed, variation derived from tissue culture (somaclonal variation) has become a phenomenon encountered by all nurseries using tissue culture as a means of propagation (Skirvin *et al.*, 1994). According to Scowcroft (1985), clonal uniformity is now recognized as the exception, rather than the rule. While these drawbacks may paint a rather bleak picture of micropropagation through tissue culture, Smith and Drew

(1990) still maintain that significant opportunities exist for the application of tissue culture in plant propagation and Smith (1994) states that the clonal mass propagation of plants *in vitro* has had the strongest practical impact on the commercial plant production industries of all tissue culture techniques. In fact, the discovery of somaclonal variation, and its manipulation through the application of *in vitro* stresses and selections, has provided plant breeders with an extremely powerful tool which could be used to great effect in the avocado industry.

1.2.2.2 Breeding

The production of novel genotypes through tissue culture directly, via somaclonal variation, or indirectly through associated technologies such as *Agrobacterium*-mediated mutation, biolistics and protoplast transformation, is becoming increasingly important to plant breeders. Avocado growers, as mentioned before, are heavily reliant on only a handful of elite scion and rootstock cultivars and, although genetically diverse (Davis *et al.*, 1998), the inherent evolutionary traits that characterize *P. americana* (i.e. high heterozygosity, an outbreeding nature and a long juvenile phase), have hindered conventional breeding efforts. Somatic cell genetics and molecular approaches can find application in almost every area of avocado breeding and selection.

Perhaps the largest threat, at present, to avocado growers worldwide is the continued lack of a rootstock that is resistant to the root rot fungus *Phytophthora cinnamoni*. The genus *Persea* contains two subgenera, *Persea*, of which the cultivated avocado is a member, and *Eriodaphne* (Scora and Bergh, 1990). Within the subgenus *Eriodaphne*, are two species, *Persea borbonia* and *P. caerulea*, which show total resistance to *P. cinnamoni* (Pliego-Alfaro and Bergh, 1992). Inter-subgeneric hybridization between these two species and commercial cultivars, however, has proven to be unsuccessful as has their use as grafted rootstocks (Pliego-Alfaro and Bergh, 1992). Tissue culture offers two technologies which may be applicable to the development of a hybrid clone or a graft-compatible selection with increased

resistance. Firstly, the technique of embryo rescue has been used to recover plants during attempts at wide hybridization by sexual crosses between distantly related plants (Williams *et al.*, 1982). Success has been obtained with interspecific crosses in agronomic (cotton, barley and rice), vegetable (cabbage) and fruit (tomato and melon) crops, and with intergeneric crosses such as barley x rye and wheat x rye (Raghavan, 1976 (cited by Smith and Drew, 1990)). Protoplast fusion, or somatic hybridization is another technique which could be used to incorporate resistance from *Eriodaphne* into *P. americana* and/or infer graft compatibility with commercial avocados. Ochatt (1990) reported on a somatic hybrid clone between the sexually incompatible rootstocks wild pear (*Pyrus communis* var *pyraster* L., (*Pomoideae*)) and 'Colt' cherry (*Prunus avium* x *pseudocerasus* (*Prunoideae*)) which was graft compatible with members of both *Pomoideae* and *Prunoideae*. Somatic hybridization in *Citrus* is well developed and has become a model for the potential application of this technique to woody plant cultivar development (Gmitter *et al.*, 1992). No less than 15 interspecific hybrids, 7 intergeneric hybrids from sexually compatible parents, and 4 intergeneric hybrids from sexually incompatible parents have been produced. The large majority of these are being evaluated, not as potential scions, but as improved rootstock selections with regard to growth habit, scion quality and abiotic and biotic stress tolerance/resistance (Grosser and Gmitter,1990).

The selection of mutants with increased resistance to various diseases through somaclonal variation has been documented for several species (Maluszynski *et al.*, 1995), the most important of which (with regard to avocado) are perhaps potato (vs. *Phytophthora infestans*)(Behnke, 1979; 1980) and peach (vs. *Xanthomonas campestris* pv. *pruni*)(Hammerschlag, 1988). In both cases, callus cultures were subjected to selection pressures in the form of toxic disease culture filtrates. Calli, capable of growing under these conditions, were isolated and manipulated to regenerate plants with acquired disease resistance. Recovery of somaclonal variants from callus or cell cultures of avocado using this approach could be a way of obtaining material with increased resistance to *P. cinnamoni*.

Two other methods with potential application to disease and pest control in avocado are mentioned by Pliego-Alfaro and Bergh (1990). Both approaches require gene isolation and gene insertion, the latter operation being accomplished through an intermediate tissue culture phase. The first method requires the isolation and introduction into the plant, of a gene encoding a phytoalexin, which efficiently controls the fungus. Transgenic tobacco with resistance against *Botrytis* has been produced using this method although the performance of the crop was not evaluated (Hain *et al.*, 1990). The second method involves protection against lepidopterans (in avocado, *Amorbia cuneana*, *Sabulodes aegrotata* G. and *Cryptoblades Gnidiella* M.) by the insertion of genes isolated from *Bacillus thuringiensis* into plant tissue. Vaeck *et al.* (1987) and Delanney *et al.* (1989) using tobacco and tomato respectively, reported resistance to lepidopterans in transgenic plants.

While the search for *Phytophthora* resistant selections is universal, particular industries have different breeding priorities in terms of tree performance. Selection pressure *in vitro* or transformation via gene insertion can, again, be used to enhance breeding programmes. For example, salinity tolerance is of major concern in Israel, California and some parts of Australia (Ben-Ya'acov and Michelson, 1995). The application of *in vitro* selection techniques could prove useful in this case as has been shown in *Citrus* (Piqueras *et al.*, 1994), *Brassica juncea* L. (Jain *et al.*, 1991) and rice (Zapata *et al.*, 1991). Other areas with potential for tissue culture-enhanced breeding techniques include tree size control, cold hardiness, fruit size and a longer marketing period achieved with later maturing fruit (Pliego-Alfaro and Bergh, 1992).

As consumers worldwide become more aware of the benefits of essential mineral and organic nutrients to their health and longevity, there is a growing trend towards the use of basic, natural ingredients. Since plants form the foundation of the human dietary food chain, these essential compounds are either directly or indirectly derived from them. Studies over the last 20 – 30 years have indicated that the inclusion of various vitamins, minerals and phytochemicals in one's diet significantly reduces the risk of chronic diseases such as cancers, cardiovascular complications and degenerative conditions

associated with aging (Bergh, 1991a and b; Grusak and Dellapena, 1999). The increasing interest in this area of nutrition has led plant molecular biologists to investigate the underlying genetic, biochemical and physiological mechanisms involved in the biosynthesis of various essential organic compounds as well as the uptake, transport and storage of nutrient minerals. Assimilation and interpretation of these results allows plant breeders to potentially manipulate nutrient composition and density in plants (Grusak and Dellapena, 1999).

According to Bergh (1991a; b), the avocado is a nutrient dense fruit providing four important minerals (iron, magnesium, potassium and copper) and seven essential vitamins (vitamins A, C, E and B_6, folacin, niacin and pantothenic acid) in an approximately 2:1 calorie ratio. In addition, the fruit has an exceptionally high fat content, which can reach over 20% of the fresh weight in some cultivars (Biale and Young, 1971). While cultivar differences in lipid composition do exist, four common fractions have been identified: 1) neutral lipids (tri-, di- and monoglycerides) 2) phospholipids 3) glycolipids and 4) free fatty acids (Seymour and Tucker, 1993). The fatty acids oleic, linoleic, palmitic and palmitoleic are the major acids present in each lipid fraction. Significantly, 82% of the total lipid composition in the oil-rich 'Hass' cultivar is monounsaturated fat and the remainder is made up of 8% polyunsaturated and 10% saturated fat (Bergh, 1991b). The monounsaturated fatty acids (oleic and palmitoleic) have been implicated in actively reducing the levels of toxic low density lipoproteins (LDL) while at the same time protecting the high density lipoproteins (HDL) which have been shown to decrease cholesterol (Assman and Schreiver, 1980). Polyunsaturated fats (in avocado, linoleic and linolenic) have also been shown to decrease LDL but, after a threshold level tend to also decrease the levels of the protective HDL. Nevertheless, these two polyunsaturated fatty acids cannot be synthesized by humans and must thus be obtained from a plant source (Grusak and Dellapena, 1999). The existence of such a rich source of essential compounds in a fruit that is already consumed worldwide presents an ideal natural system that molecular biologists and plant breeders can exploit.

The improvement of plant nutrient composition as suggested above, however, is not without its limitations. For instance, the energy cost to the plant to noticeably increase the content of plant nutrients already present in relatively high concentrations (mg), such as macronutrients and, in the case of avocado, monounsaturated fats, would be too high. However, the levels of essential elements and compounds present in small amounts (μg) are more conducive to manipulation because of a lower energy cost. In avocado, this means that compounds such as the vitamins A, C and E, for example, could be targeted for manipulation. With respect to this, Grusak and Dellapenna (1999) briefly reviewed an improvement strategy for vitamin E which would be particularly applicable to avocado. The vitamin E content of extracts of avocado oil, while comparatively high on a per calorie basis, is only between 0.8 IU and 4.2 IU (Human, 1987). The value of vitamin E to human health cannot be over emphasized. Its antioxidant properties have made it an integral component of many skin care products and dietary supplements. In nutrition, vitamin E has been shown to reduce heart disease by decreasing blood LDL oxidation that leads to plaque deposits in arteries and, in addition, the risk of cancer is believed to be reduced by an adequate intake of vitamin E (Bergh, 1991b).

Vitamin E is comprised of a group of lipid soluble antioxidants known as the tocopherols which are only synthesized by photosynthetic organisms (Grusak and Dellapenna, 1999). Four tocopherols have been identified: α-, γ-, β- and δ-tocopherol. Of these, α-tocopherol possesses the highest *in vivo* antioxidant properties and, in early work was identified as having the highest vitamin E activity (Evans and Bishop, 1922 (cited by Grusak and Dellapenna, 1999); Fukuzawa *et al.*, 1982 (cited by Grusak and Dellapenna, 1999)). The biosynthetic pathway of tocopherols in higher plants is presented in Fig. 1.2. To increase the tocopherol content in plant tissues, two hypothetical methods have been proposed. Firstly, the total tocopherol content could be increased. This would involve manipulating carbon flux through the pathway and would require genetic modification of multiple enzymatic activities. While ultimately desirable, present gene insertion and modification techniques are too clumsy

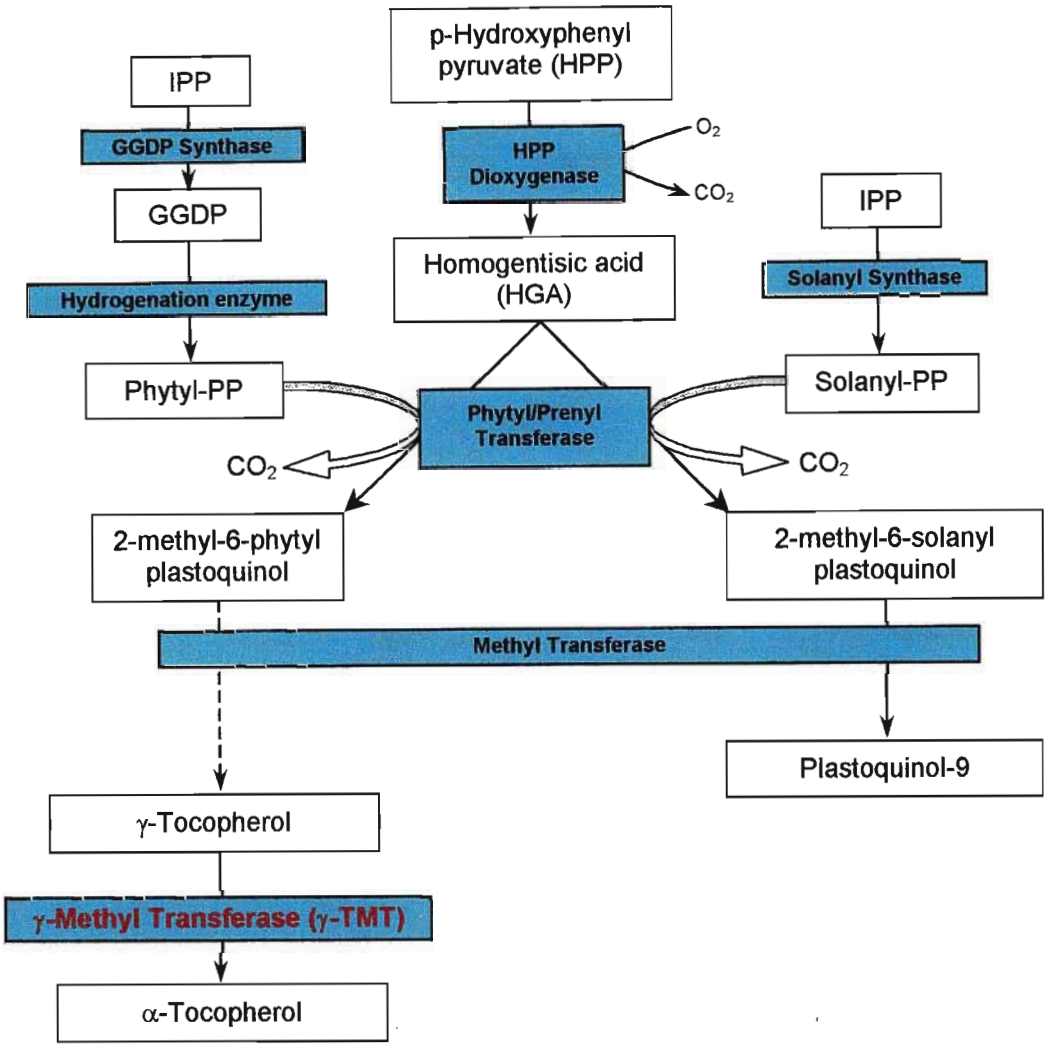


Figure 1.2

Tocopherol biosynthetic pathway. Enzymatic activities are presented against a blue background. The postulated rate limiting step for α-tocopherol production in oil seed crops is catalyzed by γ-tocopherol methyl transferase (γ-TMT). HPPDase is generally accepted as having a cytosolic localization while all other enzymes are presumably localized to plastids (after Grusak and Dellapenna, 1999).

for delicate alterations of multiple enzymatic functions. The second method involves the manipulation of the total tocopherol composition such that α-tocopherol is preferentially produced. This requires the identification of rate-limiting steps in the pathway and subsequent alterations of enzymatic activities at these points (Grusak and Dellapenna, 1999). It has been suggested that the methylation of γ-tocopherol to α-tocopherol, which is catalyzed by the γ-tocopherol methyl transferase enzyme (γ-TMT) is rate

limiting in the seeds of many important oil crops (e.g. soybean). To test this hypothesis, cloned γ-TMT cDNA was overexpressed on a seed specific promoter in *Arabidopsis*. Pooled, segregating T2 seeds from primary transformants were analysed for alterations in tocopherol content and composition. Several lines overexpressing γ-TMT were isolated and analyses showed that 85 – 95% of their total tocopherol pool was α-tocopherol, an increase of more than 80-fold over the wild-type *Arabidopsis* controls (±1% α-tocopherol) while total tocopherol contents were unchanged (Grusak and Dellapenna, 1999). This investigation illustrates the incredible potential of gene modification for improving the nutritional composition of plants and, while application of this technology to fruit is still many years away, its recognition by molecular biologists and plant breeders is important.

In this area of research, the application of tissue culture techniques may be very useful. The elucidation of biochemical pathways, the insertion of genes and the production and analysis of products may all be achieved *in vitro*. In addition, the production of transgenic plants after transformation *in vitro* will be especially important with regard to perennial fruit crops. The development of cell cultures with the ability for increased production of essential compounds as well as non-essential phytochemicals with health benefits presents a great opportunity with far reaching commercial gains. For avocado, these prospects are amplified even further since it is already such a nutrient dense fruit.

1.2.2.3 Research

The third area of avocado production in which tissue culture can be applied is that of research. Plant tissue culture presents a unique research method for in-depth physiological and biochemical analyses of direct plant responses to environmental, biotic and abiotic conditions that would be obscured *in vivo*, in complex growing environments (Smith, 1994). Cell, callus and protoplast cultures can be used to investigate the level of receptors and ultrastructural changes or to elucidate biochemical pathways under reproducible, defined and adjustable physical and chemical microenvironmental conditions (Smith,

1994). Further, perennial fruit crops are subject to seasonal fluctuations in supply. The establishment and maintenance of germplasm collections in the form of callus or cell cultures offers direct access to particular varieties by researchers at any time of year.

Of particular interest to the South African avocado industry is the investigation into the phenomenon of small fruit in the 'Hass' avocado. Two distinct populations of fruit, small and normal, are produced by 'Hass' avocado trees (Zilkah and Klein, 1987). While cell number has been recognized as the limiting parameter for growth in the small fruit phenotype, the causal mechanism of reduced cell division remains unresolved. Significant progress has been made, however, and it has been postulated (Moore-Gordon *et al.*, 1998) that the small fruit phenotype is a function of the expression of a multitude of signaling pathways seemingly initiated by a reduction in the cytokinin (CK) : abscisic acid (ABA) ratio in response to abiotic/biotic stress (Cowan *et al.*, 1997; Moore-Gordon *et al.*, 1998). As the above infers, the problem is complex and cannot be attributed to a single pathway or sequence of biochemical events at present. Nevertheless, it has been observed that an elevated ABA level in the fruit is consistent with the appearance of the small-fruit variant. Obviously this will disturb cellular balance through a cascade of biochemical and physiological events of which we know very little. At least four responses to the increased level of ABA have been suggested which may result in the symptoms observed in the small fruit. First, there is evidence to suggest that ABA acts directly to reduce cell division through the retardation of cell cycle activity (Cowan *et al.*, 1997). Second, it was postulated (Cowan *et al.*, 1997) that perturbation of the isoprenoid pathway at the level of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) by the initiation of the ABA signal transduction pathway resulted in decreased phytosterol and CK biosynthesis. Phytosterols are involved in maintaining lipid membrane integrity (Hartmann and Benveniste, 1987; Stillwell *et al.*, 1990) while ABA has been shown to increase the permeability of lipid membranes (Stillwell *et al.*, 1989). The reduction in phytosterol biosynthesis with the concurrent accumulation of ABA may result in the onset of senescence (of the seed coat) or apoptosis (programmed cell death) and cessation of fruit development. Third, it has

been suggested (Koch, 1996) that a decline in the CK:ABA ratio may influence the supply of photoassimilate required for cell division and differentiation particularly as the induction of mitosis and protein synthesis is thought to require sucrose. In addition, elevated insoluble acid invertase levels (responsible for the hydrolysis of sucrose in the apoplast) have been found in the seed of small fruit (Richings *et al.*, 2000 (in press)). Besides being implicated in reduced plant organ growth (Dickinson *et al.*, 1991; Klann *et al.*, 1996), this increase in insoluble acid invertase suggests that some change has occurred in the sucrose gradient between the mesocarp, seed coat and seed. A shift in the sucrose gradient between these tissues would result in an alteration of sink strength and therefore fruit growth and development (R. F. Cripps and A. K. Cowan, unpublished data). Fourth, Moore-Gordon *et al.* (1998) suggested that there was a connection between elevated ABA levels and the deposition of globular plasmodesmatal-localized material which blocked molecular symplastic solute transport in the mesocarp and seed coat of small fruit. In addition, it was shown that plasmodesmatal branching, evident in normal fruit and characteristic of dividing cells, was reduced or absent in small fruit (Moore-Gordon *et al.*, 1998). In avocado, the maintenance of symplastic continuity throughout fruit development is necessary for normal ontogeny because cell division occurs from fruit set to maturity albeit at a lower rate during maturation. The blocking of plasmodesmata interrupts symplastic movement and may contribute to early seed coat senescence and cessation of fruit growth.

The investigation into the occurrence of the small-fruit phenotype in 'Hass' avocado is by no means complete, and the above description is but a brief synopsis of results obtained thus far. The four responses to elevated ABA levels are not isolated pathways but are highly integrated and complex. The application of tissue culture to such a problem provides a system whereby inter- and intracellular responses can be investigated and quantified under defined conditions providing more detailed and reproducible results than those that might be obtained *in vivo*.

1.3 EPILOGUE

In conclusion, the development of a protoplast-to-plant system for *P. americana* would provide a much needed technology. Application of tissue culture to avocado propagation and breeding have tremendous potential but first require the development of an efficient and reproducible protocol for plant regeneration via both direct and indirect organogenesis and embryogenesis, a feat that has remained elusive. Tissue culture application in avocado research, on the other hand, offers immediate possibilities but for some reason has been underutilized. It is proposed that through the use of *in vitro* systems such as callus, cell and protoplast cultures in crop research that the key(s) to reversibility of recalcitrance in avocado will be uncovered. The development of model systems for research into biochemical and physiological phenomena is an important step towards bringing the knowledge and manipulation of the avocado up to a level similar to that of other perennial fruit crop species.

1.4 OBJECTIVES

The objectives of this research project are:

- To investigate the potential for protoplast isolation and culture from fruit tissue, and the generation of cell suspension cultures for future use in genetic studies
- To initiate and maintain avocado cell cultures for use in metabolic studies specifically with regard to fruit growth, ripening etc.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

2.1.1 Enzymes and fine chemicals

Hemicellulase and pectinase (from *Aspergillus niger*) were purchased from Sigma, St. Louis, Missouri. Cellulase (from *Trichoderma viride*) was purchased from Boehringer Mannheim, Germany.

Indole butyric acid (IBA), α-naphthalene acetic acid (NAA), 6-benzylaminopurine (BA), 6-furfurylaminopurine (Kn) and 6-(γ,γ-dimethylallylamino)-purine (iP) were purchased from Sigma, St. Louis, Missouri. 2,4-dichlorophenoxyacetic acid (2, 4-D) was purchased from BDH Chemicals, Poole, England.

Phenosafranin, acridine orange (AO) and fluorescein diacetate (FDA) were purchased from Sigma, St. Louis, Missouri.

Mevastatin (compactin), DL-mevalonic acid lactone (MVL), farnesyl diphosphate (FDP) and 3β-hydroxy-24-ethyl-5,22-cholestadiene (stigmasterol) were purchased from Sigma, St. Louis, Missouri.

2.1.2 Sugars

Fructose, sucrose, glucose, sorbitol and D-mannitol were purchased from Associated Chemical Enterprises (ACE), Glenvista, South Africa. D(+)-xylose, L(+)-rhamnose monohydrate and cellobiose were purchased from Merck, Germany. D(-)-ribose and D(+)-mannose were purchased from Sigma, St. Louis, Missouri.

2.1.3 Prepared media

Kao and Michayluk (1975) protoplast culture mineral salts, organic acids and vitamins were purchased as prepared media from Sigma, St. Louis, Missouri.

2.1.4 General chemicals

2(N-morpholino) ethane sulphonic acid (MES) and 1,4-dithiothreitol (DTT) were purchased from Boehringer Mannheim, Germany. Polyethylene glycol 6000 (PEG) was purchased from Merck, Germany. Nicotinic acid, *myo*-inositol, thiamine HCl, pyroxidine HCl, bovine serum albumin (BSA), agar, agarose, casein hydrolysate, ficoll 400 and coconut water were purchased from Sigma, St. Louis, Missouri.

2.2 PREPARATION OF MEDIA

2.2.1 Protoplast isolation

Five media were used for the isolation of protoplasts from 'Hass' avocado mesocarp tissue and cell suspensions. Media components and compositions were based on selected references and are presented in Tables 2.1 through 2.5.

2.2.1.1 Chapman, Power and Wilson (CPW) salts (Power *et al.*, 1984)

Table 2.1

Media components of CPW salts used to isolate protoplasts from mesocarp tissue and cell suspensions of 'Hass' avocado

Stock	Component	Company	Mass/100mL stock (mg)	Vol of stock /L final soln (mL)	Final conc (mgL^{-1})
A	$CaCl_2.2H_2O$	Holpro Analytics	4400	10	440
B	KH_2PO_4	Holpro Analytics	272	10	27.2
	KNO_3	Holpro Analytics	101		10.1
	$MgSO_4.7H_2O$	Holpro Analytics	246		24.6
	KI	BDH Chemicals	0.16*		0.016
	$CuSO_4.5H_2O$	Holpro Analytics	0.25**		0.025
	pH				5.6

*,** Stock solutions of 1.6 $mgmL^{-1}$ (KI) and 2.5 $mgmL^{-1}$ ($CuSO_4.2H_2O$) were prepared for these two solutions from which 100 µL was added to stock solution B.

To this media, the following protease substrate and antioxidant were added after the method of Percival *et al.* (1991):

- Bovine serum albumin (BSA) 0.5%
- 1,4-dithiothreitol (DTT) 0.1 mM

In addition, several concentrations of mannitol and sucrose were used during protoplast isolation and purification. These solutions, for ease of reference in later sections, were assigned abbreviations according to the concentration (in percentage weight per volume) of the particular sugar and are listed below with explanations.

CPW9M – CPW salts with 9% mannitol

CPW13M – CPW salts with 13% mannitol

CPW25S – CPW salts with 25% sucrose

2.2.1.2 Citrus protoplast isolation medium (CPIM) (Grosser and Chandler, 1987)

Table 2.2

Media components of CPIM salts used to isolate protoplasts from cell suspensions of 'Hass' avocado

Stock	Component	Company	Mass/100mL stock (mg)	Vol of stock /L final soln (mL)	Final conc (mgL^{-1})
	$CaCl_2.2H_2O$	Holpro Analytics			176
	$NaH_2PO_4.2H_2O$	Holpro Analytics			22
	MES*	Boehringer			117
	D-Mannitol	ACE			12750
	pH				5.6

*MES – 2(N-morpholino)ethane sulphonic acid

2.2.1.3 Carrot protoplast isolation medium (CtPIM) (Constabel, 1982)

Table 2.3

Media components of CtPIM salts used to isolate protoplasts from cell suspensions of 'Hass' avocado

Stock	Component	Company	Mass/100mL stock (mg)	Vol of stock /L final soln (mL)	Final conc (mgL^{-1})
	$CaCl_2.2H_2O$	Holpro Analytics			10
	$NaH_2PO_4.2H_2O$	Holpro Analytics			10
	D-Mannitol	ACE			5000
	Sorbitol	ACE			5000
	pH				5.5

2.2.1.4 Sorghum protoplast isolation medium (SPIM) (Constabel, 1982)

Table 2.4

Media components of SPIM salts used to isolate protoplasts from cell suspensions of 'Hass' avocado

Stock	Component	Company	Mass/100mL stock (mg)	Vol of stock /L final soln (mL)	Final conc (mgL^{-1})
	$CaCl_2.2H_2O$	Holpro Analytics			10
	$NaH_2PO_4.2H_2O$	Holpro Analytics			10
	Sorbitol	ACE			10000
	pH				5.5

2.2.1.5 Avocado protoplast isolation medium (APIM) (Blickle *et al.*, 1986)

Table 2.5

Media components of APIM salts used to isolate protoplasts from cell suspensions of 'Hass' avocado

Stock	Component	Company	Mass/100mL stock (mg)	Vol of stock /L final soln (mL)	Final conc (mgL^{-1})
	D-Mannitol	ACE			0.7M
	PEG*	Merck			0.4%
	pH				5.6

*PEG – Polyethylene glycol 6000

2.2.2 Protoplast culture

The most successful protoplast culture medium according to Grosser (1994) is that of Kao and Michayluk (1975). It is a highly defined medium consisting of a variety of mineral salts, sugars, organic acids, vitamins and hormones essential for multiplication and survival and can be incorporated into a liquid, agar or agarose medium. The media is abbreviated to KP8 and its composition is presented in Table 2.6.

Table 2.6

Media components of KP8 used for the culture of protoplasts isolated from 'Hass' avocado mesocarp tissue

Stock	Component	Company	Mass/100mL stock (mg)	Vol of stock /L final soln (mL)	Final conc (mgL^{-1})
Mineral salts	$CaCl_2.2H_2O$	Sigma		200	600
	NH_4NO_3				600
	KNO_3				1900
	KI				0.75
	KCl				300
	$CoCl_2.6H_2O$				0.025
	KH_2PO_4				170
	H_3BO_3				3
	$Na_2MoO_4.2H_2O$				0.25
	$MgSO_4.7H_2O$				300
	$MnSO_4.H_2O$				10
	$CuSO_4.5H_2O$				0.025
	$ZnSO_4.7H_2O$				2
	$FeSO_4.7H_2O$				28
Sugars A	Fructose	ACE	125	100	125
	D(-)-Ribose	Sigma	125		125
	D(+)-Xylose	Merck	125		125
	D(+)-Mannose	Sigma	125		125
	L(+)-Rhamnose monohydrate	Merck	125		125
	Cellobiose	Merck	125		125
	Sorbitol	ACE	125		125
	(D)-Mannitol	ACE	125		125
Sugars B	Glucose	ACE			68400
	Sucrose AR	ACE			250
Organic acids	Sodium pyruvate	Sigma		$70mgL^{-1}$	5
	Citric acid				10
	Malic acid				10
	Fumaric acid				10

Table 2.6 continued.....\

Stock	Component	Company	Mass/100mL stock (mg)	Vol of stock/L final soln (mL)	Final conc (mgL^{-1})
Vitamins	Inositol	Sigma		10	100
	Nicotinomide				1
	Pyridoxine-HCl				1
	Thiamine-HCl				10
	D-Calcium pantothenate				0.5
	Folic acid				0.2
	p-Aminobenzoic acid				0.01
	Biotin				0.005
	Choline chloride				0.5
	Riboflavin				0.1
	Ascorbic acid				1
	Vitamin A				0.005
	Vitamin D_3				0.005
	Vitamin B_{12}				0.01
Hormones	IBA	Sigma	50	1.2	0.6
	NAA	Sigma	50	1.2	0.6
	2.4D	BDH Lab. Chemicals	50	2.0	1.0
	6BAP	Sigma	50	0.6	0.3
	Kn	Sigma	50	0.6	0.3
	2Ip.	Sigma	50	0.6	0.3
Casein hydrolysate		Sigma			125
Ficoll 400		Sigma			10000
MES		Boehringer Mannheim			3904
Coconut water		Sigma			10mL

2.2.3 Callus induction and cell suspension culture

The media developed by Murashige and Skoog (1962) (MS) and Gamborg *et al.* (1968) (B5) have been used extensively for callus culture on agar as well as cell suspension cultures in liquid media with varying degrees of success depending on the species being cultured. Table 2.7 and 2.8 show the components of the MSB medium (MS basal salt media with B5 vitamins) that was used for callus and cell cultures.

Table 2.7

Media components of MS basal salt medium used for the induction and maintenance of callus and cell cultures from 'Hass' avocado seed, mesocarp and embryo tissue

Stock	Component	Company	Mass/100mL stock (mg)	Vol of stock /L final soln (mL)	Final conc (mgL^{-1})
A	$CaCl_2.2H_2O$	Holpro Analytics	4400	10	440
B	NH_4NO_3	ACE	16500	10	1650
	KNO_3	Holpro Analytics	19000		1900
C	KI	BDH Chemicals	8.3	10	0.83
	$CoCl_2.6H_2O$	NT Lab. Supplies	0.25*		0.025
D	KH_2PO_4	Holpro Analytics	1700	10	170
	H_3BO_3	Holpro Analytics	62		6.2
	$Na_2MoO_4.2H_2O$	ACE	2.5		0.25
E	$MgSO_4.7H_2O$	Holpro Analytics	3700	10	370
	$MnSO_4.4H_2O$	Hopkin and Williams	223		22.3
	$CuSO_4.5H_2O$	Holpro Analytics	0.25**		0.025
	$ZnSO_4.7H_2O$	Holpro Analytics	86		8.6
F	$FeSO_4.7H_2O$	SAARchem	557	10	55.7
	Na_2EDTA	SAARchem	745		74.5

*,** Stock solutions of 250 mg/100 mL ($CoCl_2.6H_2O$) and 250 mg/100 mL ($CuSO_4.2H_2O$) were made up for these two solutions from which appropriate volumes were added to the stock solutions.

Table 2.8

Media components of B5 vitamin media used to supplement MS salts for the induction and maintenance of callus and cell cultures from 'Hass' avocado seed, mesocarp and embryo tissue

Stock	Component	Company	Mass/100mL stock (mg)	Vol of stock /L final soln (mL)	Final conc (mgL^{-1})
G	*myo*-Innositol	Sigma	10000	1	100
	Nicotinic acid	Sigma	100		1
	Thiamine HCl	Sigma	1000		10
	Pyroxidine HCl	Sigma	100		1

For solid and liquid culture, 3% sucrose was added to MSB as an organic carbon source and the pH was adjusted to 5.8 using HCl (0.1 N). Media for callus induction and maintenance was solidified with 0.8% agar.

All media (excepting that containing enzymes and protein substrate), glassware and instruments were sterilised in a HL-300 Huxley autoclave

(Hung Lin Medical Instruments, Taiwan) at 1.5 kPa and 127°C for 10 min. Media for the isolation of protoplasts, which contained enzymes and protein substrate, was sterilised by filtering through a 0.22 μm Millipore® nylon filter housed in a Swinnex-21 (Millipore Corporation, Bedford) filter unit and attached to a sterile glass syringe. Unless otherwise stated, all procedures were carried out in a laminar flow cabinet using normal aseptic technique.

2.3 PREPARATION OF GROWTH REGULATORS, ISOPRENOIDS AND STAINS

0.5 $mgmL^{-1}$ stock solutions of hormones were prepared by dissolving 50 mg of auxin or cytokinin in 2 mL 0.2 N NaOH or 0.2 N HCl respectively, and making up to 100 mL. The stock solutions were stored at 4°C and were freshly prepared every month.

A stock solution of mevastatin was prepared by dissolving 5 mg mevastatin in 10 mL dimethyl sulfoxide (DMSO). This 0.5 $mgmL^{-1}$ stock solution was diluted as and when required.

For isoprenoids, a 1 mM stock solution of farnesyl diphosphate (FDP) was prepared by diluting 100 μL of a 10 mM solution to 1 mL with DMSO. 100 mg of DL-mevalonic acid lactone (MVL) was dissolved in 1 mL DMSO to give a 100 $mgmL^{-1}$ stock solution, while a 1 $mgmL^{-1}$ stock solution of stigmasterol was made by dissolving 10 mg in 10 mL ethanol.

The stains, phenosafranin, acridine orange, fluorescein diacetate (FDA) and fluorescent brightener 28 (syn. calcofluor white) were prepared as 1 $mgmL^{-1}$ stock solutions in water (phenosafranin and acridine orange), DMSO (fluorescent brightener 28) and acetone (FDA).

2.4 PLANT MATERIAL

For protoplast isolation, cell and callus culture, fruit from the Guatemalan x Mexican hybrid cultivar 'Hass' were used. The fruit were obtained from two commercial farms, Everdon Estate and Bounty Farm, both situated in the Kwa-Zulu Natal midlands (Philips bioclimatic group 3 – cool subtropical, summer rainfall areas). The trees on Everdon Estate from which fruit were harvested, had been grafted onto clonal 'Duke 7' rootstock and were planted in 1988. Fruit from Bounty Farm were harvested from 8 yr old trees (in 1998) also growing on clonal 'Duke 7' rootstock.

Fruit used for all experiments were washed with concentrated Extran® detergent (Merck, Germany) and placed in concentrated Jik® commercial bleach (3.5% sodium hypochlorite) with several drops of Tween 20. After 15 min, the fruit was removed and placed in 80% ethanol for 1 min. The fruit was then rinsed three times with ultra pure water.

2.5 PROTOPLAST ISOLATION FROM THE MESOCARP OF 'HASS' AVOCADO FRUIT

A core of yellow mesocarp tissue was removed from a decontaminated fruit with a No. 6 cork borer and sliced into disks 0.5 mm to 1 mm thick with a sterile razor blade. The average fresh weight of five disks was determined for yield data (number of protoplasts / gram fresh weight of tissue). Disks were then placed in 5 mL of predigestion solution (see below) in a 60 x 15 mm sterile petri dish and left to plasmolyse for 45 min. After this adjustment period, the disks were removed and placed in 5 mL of digestion solution containing one of three enzyme concentrations below:

- ES1 – 0.25% (w/v) hemicellulase, 0.25% (w/v) cellulase, 0.006% (w/v) pectinase
- ES2 – 0.5% (w/v) hemicellulase, 0.5% (w/v) cellulase, 0.01% (w/v) pectinase

- ES3 – 1% (w/v) hemicellulase, 1% (w/v) cellulase, 0.02% (w/v) pectinase

The dishes were sealed and incubated in the dark at room temperature for 20 h. The predigestion solution contained CPW salts with 5% BSA, 0.1 mM DTT and 0.6 M mannitol with no enzymes. The digestion solution was the same as the predigestion solution but with the various enzymes added. After incubation the disks were teased apart to release the protoplasts and the suspension was filtered through a 150 μm nylon mesh into a sterile petri dish.

The filtrate was transferred to a 15 mL centrifuge tube and centrifuged for 10 min at low speed in a fixed angle SC-158T centrifuge (Yih Der Instruments, Taiwan). Three distinct components of the suspension were visible after centrifugation - a top layer of floating protoplasts and oil droplets; a clear middle layer of digestion medium and a characteristic pellet of cell debris. The protoplasts were removed with a pasteur pipette, transferred into 3 mL teflon centrifuge tubes and washed in predigestion medium. Each suspension was centrifuged for 10 min at 1000 rpm after which they were rewashed and centrifuged at 1000 rpm for 5 min (Hitachi Himac SCT15B, Japan).

2.6 CALLUS INDUCTION

MSB medium, solidified with 0.8% agar and containing 3% sucrose was used for callus induction. Two types of culture vessel were used – a) 20 x 150 mm tissue culture vials with aluminium tops, b) 20 mL econo glass vials with screw on caps. The hormone combinations and concentrations used for each treatment are detailed below in Table 2.9.

Table 2.9

Hormone treatments used for the culture of avocado mesocarp and endosperm explants

		BA				iP		
	Conc. (mgL^{-1})	0	0.5	1.0	5.0	0.5	1.0	5.0
NAA	0	0/0	0/.5	0/1	0/5	0/.5	0/1	0/5
	0.5	.5/0	.5/.5	.5/1	.5/5	.5/.5	.5/1	.5/5
	1.0	1/0	1/.5	1/1	1/5	1/.5	1/1	1/5
	5.0	5/0	5/.5	5/1	5/5	5/.5	5/1	5/5
IBA	0.5	.5/0	.5/.5	.5/1	.5/5	.5/.5	.5/1	.5/5
	1.0	1/0	1/.5	1/1	1/5	1/.5	1/1	1/5
	5.0	5/0	5/.5	5/1	5/5	5/.5	5/1	5/5

There were 49 treatments and each treatment was replicated ten times.

Excised embryos were cultured in the same basal medium but with different hormone concentrations based on the results of Gonzalez-Rosas *et al.* (1990) as well as those from callus induction in mesocarp and endosperm explants. The treatments are shown in Table 2.10 below.

Table 2.10

Hormone treatments used for the culture of excised embryo explants

		Kinetin					iP				
	Conc. (mgL^{-1})	0.01	0.1	0.3	0.5	1.0	0.01	0.1	0.3	0.5	1.0
NAA	5.0	/	/	/	/	/	/	/	/	5/.5	/
IBA	0.3	.3/.01	.3/.1	.3/.3	.3/.5	.3/1	.3/.01	.3/.1	.3/.3	.3/.5	.3/1
	1.0	1/.01	1/.1	1/.3	1/.5	1/1	1/.01	1/.1	1/.3	1/.5	1/1
	5.0	5/.01	5/.1	5/.3	5/.5	5/1	5/.01	5/.1	5/.3	5/.5	5/1
2.4D	5.0	/	/	/	/	/	/	/	/	5/.5	/

Six to seven month old 'Hass' avocado fruit were sterilized and sliced open. The mesocarp and the endosperm of the seed were sectioned into explants weighing approximately 100 mg. Of each treatment, 5 vials were planted with mesocarp tissue and 5 with endosperm tissue. Embryos were excised from the cotyledons and placed in three 20 x 150 mm tissue culture vials for each treatment. The vials were kept under room temperature and light conditions and were monitored every week for callus formation and growth.

2.7 INITIATION AND MAINTENANCE OF CELL SUSPENSION CULTURES

MSB medium with 3% sucrose was used as a standard medium. The combinations and concentrations of growth regulators, shown in Table 2.11, were used to investigate optimum growing conditions. Each treatment was replicated.

Table 2.11

Combinations and concentrations of growth regulators used for cell culture

Treatment	Auxin	Concentration (mgL^{-1})	Cytokinin	Concentration (mgL^{-1})
1	2, 4-D	0.2	-	-
2	2, 4-D	0.5	-	-
3	2, 4-D	1	-	-
4	2, 4-D	5	-	-
5	2, 4-D	5	iP	1
6	NAA	0.2	-	-
7	NAA	1	-	-
8	NAA	5	-	-
9	NAA	5	iP	1
Control	-	-	-	-

Approximately 5 g of 'wet', friable callus, initiated from endosperm and embryo tissue was transferred to 20 mL standard media containing NAA (5 mgL^{-1}) and iP (1 mgL^{-1}) in a 250 mL Erlenmyer flask and placed on a rotary shaker at 120 rpm for 14 d. Thereafter, the cell density was determined after transfer to the treatments above to give an initial density of 5×10^7 cells mL^{-1} in 6 mL media. The experiment was carried out in 25 mL Erlenmyer flasks shaken at 120 rpm under growth room conditions (section 2.3). Cell number was measured as described in section 2.12.1.

After determining the best combination and concentration of growth regulators to use, all subsequent cultures were carried out in this media.

2.8 CULTURE CONDITIONS FOR PROTOPLASTS, CALLUS AND CELL SUSPENSION CULTURES

Unless otherwise stated, callus, embryo and cell cultures were maintained in a growth room at 27°C under a 16 h light / 8 h dark photoperiod. The room was illuminated with Philips TLD 58W/840 cool white fluorescent growth lamps producing a light intensity of 35 $\mu molm^{-2}s^{-1}$ and 7 $\mu molm^{-2}s^{-1}$ at the level of the cell and the callus cultures respectively.

For protoplast isolation, suspensions were incubated in the dark at room temperature. Cultured protoplasts were also maintained in the dark either on a shaker (120 rpm) at room temperature (agarose semi-solid drop and sector culture treatments) or in an incubator at 25°C (liquid culture and microscope slide chamber treatments).

2.9 PROTOPLAST CULTURE

Freshly isolated protoplasts were purified, resuspended in KP8 and their viability determined by the exclusion of phenosafranin. The sample population density was adjusted to three densities, a) 2.5 x 10^5 protoplasts mL^{-1} ; b) 5 x 10^5 protoplasts mL^{-1}; c) 1 x 10^6 protoplasts mL^{-1}. Each density was applied to the four culture techniques below.

A. LIQUID CULTURE: Ten 20 µL drops were placed in a 90 x 15 mm petri dish for each treatment. The dish was sealed with Parafilm® and placed in the dark at 25°C. Each treatment was replicated three times.
B. MICROSCOPE SLIDE CHAMBER: Two slides, separated by two cover slips, were sealed on three sides with molten wax. The cavity was carefully filled with protoplast suspension and the remaining side sealed. Each treatment was replicated twice and placed in the dark at 25°C.
C. AGAROSE SEMI-SOLID DROP CULTURE (after Dhir *et al.*, 1991): 0.6% agarose was used to solidify the protoplast suspension. Ten 20 µL drops were placed in 90 x 15 mm petri dishes and bathed in 5 mL KP8. The

dishes were sealed and placed on a shaker at 120 rpm under room temperature conditions. Each treatment was replicated twice.

D. AGAROSE SEMI-SOLID SECTOR CULTURE (after Dhir *et al.*, 1991): 10 mL protoplast suspension containing 0.6% agarose was pipetted into a 90 x 15 mm petri dish and left to solidify. After 3 hrs, the agarose was cut into sections and bathed in 5 mL KP8 solution. The dishes were covered in aluminium foil and placed on a shaker at 120 rpm under normal room temperature conditions. Each treatment was replicated twice.

All culture media contained the UV fluorescent stain fluorescent brightener 28 (syn. calcofluor white) at a concentration of 10 μgmL^{-1} to monitor cell wall formation.

2.10 ISOLATION OF PROTOPLASTS FROM CELL SUSPENSIONS OF 'HASS' AVOCADO

Five media were used in the isolation of protoplasts from liquid cultured cells (section 2.2.1) and are shown in Table 2.12 with their respective enzyme concentrations.

Table 2.12

Media used for isolation of protoplasts from cell suspensions

Media	Cellulase (% w/v)	Hemicellulase (% w/v)	Pectinase (% w/v)
CPW	1	1	0.1
CPIM	2	1	0.2
CtPIM	2	1	-
SPIM	1	1	-
APIM	1	-	0.2
	2	-	0.4
	4	-	1

1 mL of cell suspension was taken 4-10 d into a 14 d culture cycle and transferred to either a 60 x 15 mm petri dish (treatment CPIM) or to a 15 mL centrifuge tube (all other treatments). For isolation with CPIM, 1.25 mL of the enzyme solution was added to the cell suspension dropwise to facilitate cell separation and penetration. The petri dish was sealed, covered with

aluminium foil and placed on a rotary shaker at room temperature overnight. For the other treatments, cells were pelleted by centrifuging and the supernatant removed. The respective enzyme solutions were added as detailed below:

- CPW – 2 mL enzyme solution was added to the pelleted cells
- CtPIM and SPIM – 2 mL MSB and 2 mL enzyme solution was added to the pelleted cells
- APIM – 2 mL isolation medium without enzymes (predigestion solution) was added to the pellet. After 1 hr, 2 mL APIM with enzymes was added to the suspension.

The suspensions were transferred to 60 x 15 mm petri dishes which were sealed, covered and placed on a rotary shaker at room temperature overnight.

2.11 TREATMENT OF CALLUS AND CELL CULTURES

2.11.1 Callus subculture

Callus was subcultured every 4 weeks by aseptically removing the explant, cutting off all necrotic tissue and transferring to fresh media.

2.11.2 Callus manipulations

Callus, subcultured onto media containing a high AUX:CK ratio and maintained under continuous light, sometimes developed structures that were postulated to be somatic embryos in the globular stage of development. To encourage development of these structures they were subcultured onto several media containing various components reported to enhance further growth. The media used and any additional components are presented in Tables 2.13 and 2.14.

Table 2.13

Media components of B5 (Gamborg *et al.*, 1968) basal salt medium used for the culture of globular callus cultures

Stock	Component	Company	Mass/100mL stock (mg)	Vol of stock /L final soln (mL)	Final conc (mgL^{-1})
A	$CaCl_2.2H_2O$	Holpro Analytics	1500	10	150
B	KNO_3	Holpro Analytics	25000	10	2500
C	KI	BDH Chemicals	7.5	10	0.75
	$CoCl_2.6H_2O$	NT Lab. Supplies	0.25*		0.025
D	H_3BO_3	Holpro Analytics	30	10	3.0
	$Na_2MoO_4.2H_2O$	ACE	2.5		0.25
	$NaH_2PO_4.H_2O$	Holpro Analytics	1500		150
E	$MgSO_4.7H_2O$	Holpro Analytics	2500	10	250
	$MnSO_4.4H_2O$	Hopkin and Williams	100		10.0
	$CuSO_4.5H_2O$	Holpro Analytics	0.25**		0.025
	$ZnSO_4.7H_2O$	Holpro Analytics	20		2.0
	$(NH_4)_2SO_4$	Holpro Analytics	1340		134
F	$FeSO_4.7H_2O$	SAARchem	278	10	27.8
	Na_2EDTA	SAARchem	373		37.3
G (vitamin stock)	*myo*-Innositol	Sigma	10000	1	100
	Nicotinic acid	Sigma	100		1
	Thiamine HCl	Sigma	1000		10
	Pyroxidine HCl	Sigma	100		1

Table 2.14

Media components of Dixon and Fuller (Dixon and Fuller, 1976) basal salt medium used for the culture of globular callus cultures

Stock	Component	Company	Mass/100mL stock (mg)	Vol of stock /L final soln (mL)	Final conc (mgL^{-1})
A	$CaCl_2.2H_2O$	Holpro Analytics	2200	10	220
B	KNO_3	Holpro Analytics	25200	10	2520
C	KI	BDH Chemicals	10	10	1.0
	$CoCl_2.6H_2O$	NT Lab. Supplies	1*		0.1
D	H_3BO_3	Holpro Analytics	50	10	5.0
	$Na_2MoO_4.2H_2O$	ACE	1		0.1
E	$MgSO_4.7H_2O$	Holpro Analytics	3600	10	360
	$MnSO_4.4H_2O$	Hopkin and Williams	132		13.2
	$CuSO_4.5H_2O$	Holpro Analytics	2**		0.2
	$ZnSO_4.7H_2O$	Holpro Analytics	10		1.0
F	$FeSO_4.7H_2O$	SAARchem	557	10	55.7
	Na_2EDTA	SAARchem	745		74.5
G (vitamin stock)	*myo*-Innositol	Sigma	10000	10	1000
	Nicotinic acid	Sigma	50		5
	Thiamine HCl	Sigma	50		5
	Pyroxidine HCl	Sigma	5		0.5

*,** Stock solutions of 2.5 $mgmL^{-1}$ ($CoCl_2.6H_2O$) and 2.5 $mgmL^{-1}$ ($CuSO_4.2H_2O$) were made up for these two solutions from which appropriate volumes were added to the stock solutions.

In addition to these two solutions, MSB was diluted by half (½MSB) and used as a separate medium. Additional components to these media included activated charcoal (0.1%) and glutamic acid (400 mgL^{-1})(section 3.2.2).

2.11.3 Cell suspension subculture

Cell suspensions were subcultured every 14-21 d by transferring half of the suspension to a new flask and adding an equal volume of fresh medium to each vessel.

2.11.4 Population dynamics of 'Hass' avocado cell suspensions

To determine the contribution of the isoprenoid pathway to cell proliferation, cell cultures (5 mL) were started in 25 mL Erlenmyer flasks by adding 1 mL of a saturated suspension to 4 mL fresh MSB media containing NAA (5 mgL^{-1}) and iP (1 mgL^{-1}). The suspensions were placed on a rotary shaker at 120 rpm under growth room conditions (section 2.8). Just before the exponential phase of growth (at about 10 d), mevastatin (compactin) was added to the cultures at the concentrations specified in Table 2.13. Cells were counted every three to four days by removing 50 μL of suspension, adding 100 μL chromic acid (8% (w/v) chromic trioxide) and incubating at 70°C for 15 min. These solutions were then diluted and counted using a Sedgewick Rafter cell counting chamber (ProSciTech, Australia)(section 2.12.2). Once a week, an additional 50 μL sample was removed from each replication and pooled to create a treatment sample. This was then stained with 10 μgmL^{-1} FDA and viewed under UV (450-490 nm) light to qualitatively monitor cell viability (section 2.12.4.2).

Table 2.15

Mevastatin concentrations used to investigate cell cycle progression in avocado cell suspensions

Treatment	Concentration (μM)
A	0.01
B	0.1
C	1
D	10
E	40
Control	-

2.11.5 Reversal of mevastatin-induced cell cycle arrest by the addition of mevalonic acid lactone, stigmasterol and farnesyl diphosphate

From the preceding experiment, two mevastatin concentrations (1 μM and 40 μM) were selected to test whether products of the isoprenoid pathway could reverse the effects of mevastatin. Three isoprenoid derived compounds were added to each mevastatin concentration – DL-mevalonic acid lactone (6 mM), farnesyl diphosphate (10 μM) and stigmasterol (10 μM).

Cell cultures were started as before (section 2.10.2) and the mevastatin plus isoprenoid compounds added at day 10. Cell counting and viability testing were carried out as above.

2.12 MICROSCOPY

2.12.1 Cell counting with a hemacytometer

Population densities of protoplasts and cells in early research was determined using a hemacytometer. For this, a small volume of suspension was placed on the hemacytometer and covered with a coverslip. Cells contained in the volume of media covering the grid, were counted under a Zeiss KF2 microscope and the number corrected for dilution.

2.12.2 Cell counting with a Sedgewick Rafter counting chamber

Due to cell size and the presence of aggregates, the hemacytometer proved to be inaccurate. Cells were then counted using a Sedgewick Rafter counting chamber. 1 mL of suspension was transferred to the counting chamber and the number of cells in 20 random squares was determined under a Zeiss KF2 microscope. This number was corrected for dilution and recorded. It must be noted, however, that a random sample such as was taken here, carries with it a degree of variation that has to be countered by treatment replication. In these trials this was not always possible due to logistical constraints but it is thought that the results obtained herein, reflect to a fairly high degree of accuracy, the interactions within the cell cultures.

2.12.3 Light microscopy

Freshly isolated protoplasts were pipetted onto a microscope slide, covered with a cover slip and viewed under an Olympus BH-2 microscope (Wirsam Scientific, Durban, ZA) fitted with an Olympus C-35 AD camera (Olympus photomicrographic equipment model PM-10AD). Micrographs were taken using black and white 400ASA negative film.

2.12.4 Fluorescence microscopy

2.12.4.1 Protoplast viability

After purification, cell wall digestion was confirmed by staining the protoplast suspension with 10 μgmL^{-1} (10 μL of a 1 $mgmL^{-1}$ stock solution) fluorescent brightener 28 (syn. calcofluor white) and viewing under UV light (barrier filter: 450-490 nm; exciter filter: 510 nm; transmission filter: 520 nm) through a Zeiss Axiophot microscope. The light source for all fluorescent microscopy was a 100 W Hg vapour lamp. Treated samples were viewed after 1 min of incubation and were exposed to UV light for up to 5 min. Micrographs were taken using the fitted Axiophot camera and 400 ASA colour negative film.

Protoplast viability was measured using 3 staining techniques presented in Table 2.14.

Table 2.14
Stains used and criteria measured to determine protoplast viability

	Stain	Stain conc (μgmL^{-1})	Incubation time	UV filter		Viable cells	Non-viable cells
A	Phenosafranin exclusion	100	1min	None		Exclude phenosafranin	Stain red
B	Acridine orange fluorescence	100	1min	Barrier Exciter Transmission	450-490nm 510nm 520nm	Fluoresce green	Fluoresce red
C	Fluorescein diacetate fluorescence	10	1min	Barrier Exciter Transmission	450-490nm 510nm 520nm	Fluoresce	No fluorescence

All samples were plated on a hemacytometer (Fortuna Opticolor, Germany) and viewed through a Zeiss Axiophot microscope. Micrographs were taken using the fitted Axiophot camera and 400 ASA colour film.

2.12.4.2 Viability of cultured cells

50 μL from each replication of a treatment was removed and pooled to create a treatment sample. The sample was stained with 10 μgmL^{-1} FDA for 1 min and viewed under UV (450-490 nm) light through a Zeiss Axiophot microscope to qualitatively monitor cell viability. Samples were exposed to UV light for up to 5 min. Images were recorded using a Panasonic WV-CP450 digital camera (see section 2.13.1).

2.12.5 Scanning electron microscopy of protoplasts

For SEM work, three methods were employed for the collection of protoplasts and subsequent viewing: 1. Sedimentation on a 5 cm Nucleopore® membrane filter (10 μm pore spacing) resting on a Whatman No. 1 filter paper acting as a 'wick' to prevent drying (Hughes *et al.*, 1976), 2. Preparation in a Beem® capsule closed at both ends with 5 μm plankton mesh, 3. Preparation in a flow through capsule. Three aliquots (about 0.5 mL) of freshly isolated, concentrated protoplasts were allocated to one of the three methods above

and fixed in predigestion solution containing 3% gluteraldehyde for 1 h at room temperature. The specimens were washed twice in 0.05 M cacodylate buffer and post fixed in 2% osmium tetroxide. A graded ethanol series (30%, 50%, 70%, 80%, 90%, 100%) was used to dehydrate the protoplasts (10 min at each concentration) which were then transferred to critical point drying (CPD) baskets under 100% alcohol and dried under CPD (Hitachi HCP-2). After drying, the protoplasts from each collection method were mounted on specimen stubs and vacuum coated with gold-palladium (Polaron E5100). Specimen stubs were examined in an SEM (Hitachi S-570) at 20 kV the same day.

2.13 DATA ANALYSIS

2.13.1 Digital image acquisition and modification

Digital images for Figs. 2.1; 3.10(a – e); 3.12 (a – e); 3.14 (a, b); 3.16 (a – c) and 3.17 (a – p) were captured in JPEG file format using a Sony Mavica MVC-FD71 digital camera. Figs. 3.3 (b (i) – c (ii)); 3.4 (a, b); 3.5 (a, b); 3.20 (a – c); 4.4 and 4.6 were captured in TIFF file format using a Panasonic WV-CP450 digital camera and analySIS® 3.0 image analysis software (Soft Imaging System GmbH, Munster, Germany). Post capture modification of digital images was limited to contrast and brightness adjustment in Corel Presentations® software to enhance details. Files were saved in WPG format for printing.

2.13.2 Callus evaluation

In order to avoid destructive sampling, an arbitrary scale of 0 to 10 was developed with 0 being no callus growth, 1, the appearance of callus, 5, half the explant covered in callus and 10, no explant visible (Fig. 2.1).

2.13.3 Statistical analysis

Regression lines and r^2 values for cell suspension growth were fitted and calculated by the graphical statistical analysis tool of Microsoft Excel®. Analyses of treatment differences for cell growth responses (Chapter 4), were calculated by the descriptive data analysis function of Corel Quattro Pro®.

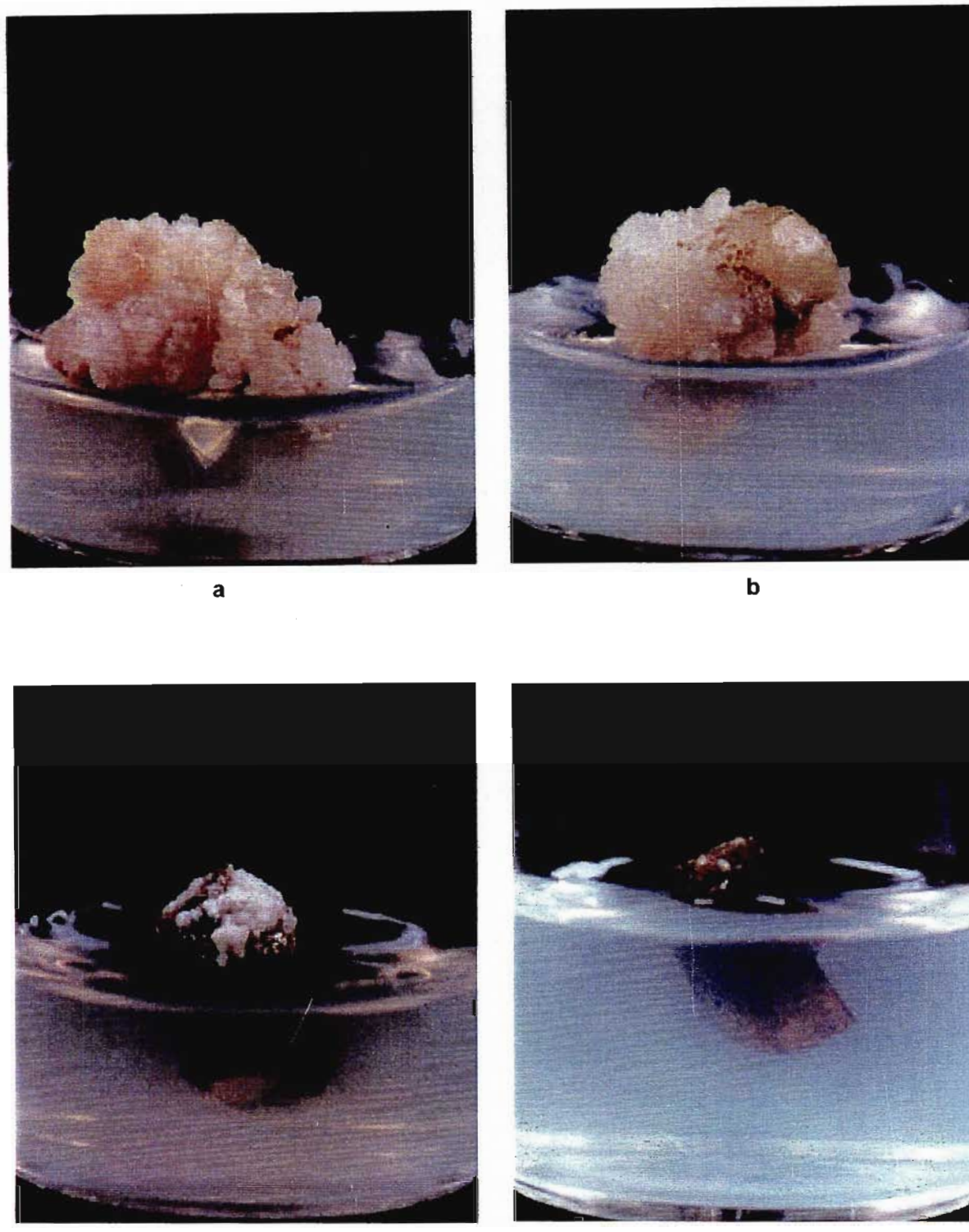


Figure 2.1

Qualitative measurement of callus growth - a = 10 (no explant visible), b = 5 (half the explant visible), c = 1 (appearance of callus), d = 0 (no callus)

CHAPTER 3

DEVELOPMENT OF AN *IN VITRO* CULTURE SYSTEM FROM AVOCADO FRUIT

3.1 INTRODUCTION

The source material for any tissue culture programme is extremely important. It is generally accepted that the explant must contain living, non-dormant cells, and that younger, more juvenile tissues, which contain a higher proportion of actively dividing cells, are more responsive to growth conditions *in vitro.* In addition, it is well known that the physiological status of the plant part exerts an influence on its response to the tissue culture programme (Collin and Edwards, 1998). The avocado fruit is unique among sub-tropical fruits in that cell division in the mesocarp proceeds throughout fruit development albeit at a lesser rate towards the end (Schroeder, 1953; Coombe, 1976). While this may be a convenient characteristic to exploit with regard to *in vitro* culture of this tissue, it is important to be aware of other physiological changes that occur over the developmental period and which may impact on the success of the programme.

During the course of development of the avocado fruit, many changes occur in both cell structure and cell physiology. Together, these changes contribute to a changing structural and physiological state at the whole fruit level. Fig. 3.1 illustrates a simplified model of avocado fruit growth and the major accompanying alterations in physiology over the developmental period. It must be stressed that all processes are not temporally separated and there is appreciable overlap between many events. Further, not all processes have been spatially identified. This means that while there is an early auxin peak, for example, on a whole fruit basis, mesocarp tissue and seed tissue may differ substantially in relative auxin content. This is also applicable on a cellular basis especially in the mesocarp where there are two cell types which differ both structurally and in lipid content. For tissue culture programmes, it is

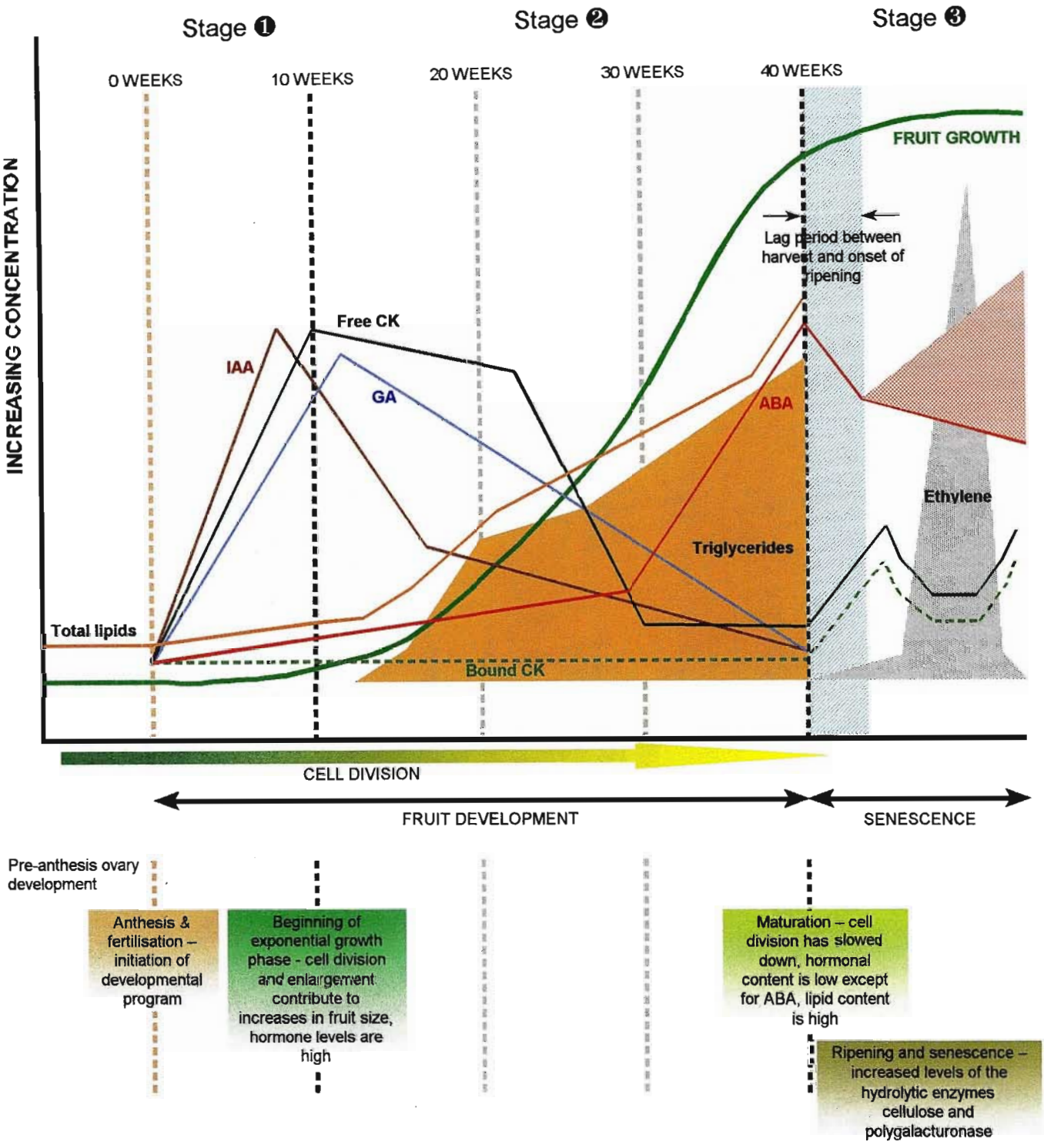


Figure 3.1

Simplified model of avocado fruit development and corresponding physiological changes with respect to plant hormone levels and lipid content. Trends in concentrations are on a whole fruit basis and it must be understood that this is not always reflective of the status of all fruit tissues at one particular time (adapted from Bower and Cutting, 1988).

important to be able to relate *in vitro* responses to the physiological state of the explant prior to planting. This allows accurate interpretation of results in the presence of confounding effects such as changing endogenous hormone levels. In this context, Fig. 3.1 provides an important and useful tool for the explanation of results.

Attention in avocado tissue culture has focussed on developing reproducible systems for plant regeneration (Mohammed-Yasseen, 1992; Mohammed-Yasseen *et al.*, 1992) and plant improvement through gene insertion (Ahmed *et al.*, 1998). However, little research has been carried out on the development of tissue culture techniques for use as model systems to study physiological and biochemical events involved in developmental processes or responses to external stimuli. There is great potential for the use of some technologies for investigations into cellular mechanisms associated with imposed stresses (both biotic and abiotic) or with different variants, for instance. In avocado, Percival *et al.* (1991) reported that protoplasts isolated from fruit mesocarp tissue had been successfully used for ripening studies and that their results reflected those obtained from intact fruit. Aside from this report, no other tissue culture technologies have been employed as vehicles for in depth research applications regarding avocado. Indeed, the literature reveals that there is an extremely high degree of variability in results between publications, and replication of experiments with comparable results is non-existent. The work of Van Llelyveld (1984) with cell suspensions, Blickle *et al.* (1986) with protoplasts from callus tissue and Percival *et al.* (1991) with protoplasts from mesocarp tissue has not been corroborated and suggests that there may be some inherent problems with the tissue culture of avocado which perhaps stem from its diverse and largely unknown genetic history.

The present investigation attempts to develop an *in vitro* technology from avocado fruit tissue for use as a model system to study biochemical aspects of fruit growth.

3.2 RESULTS

3.2.1 Protoplast isolation from mesocarp tissue

Initial results indicated that protoplasts were readily isolated from avocado mesocarp tissue at all stages of development. Yield increased linearly with increasing enzyme concentration (Fig. 3.2) and ranged from 2 x 10^7 and 8 x 10^7 protoplasts / g fresh weight. Viability, measured by the exclusion of phenosafranin, was high for all treatments being between 82% and 88% (Fig. 3.2).

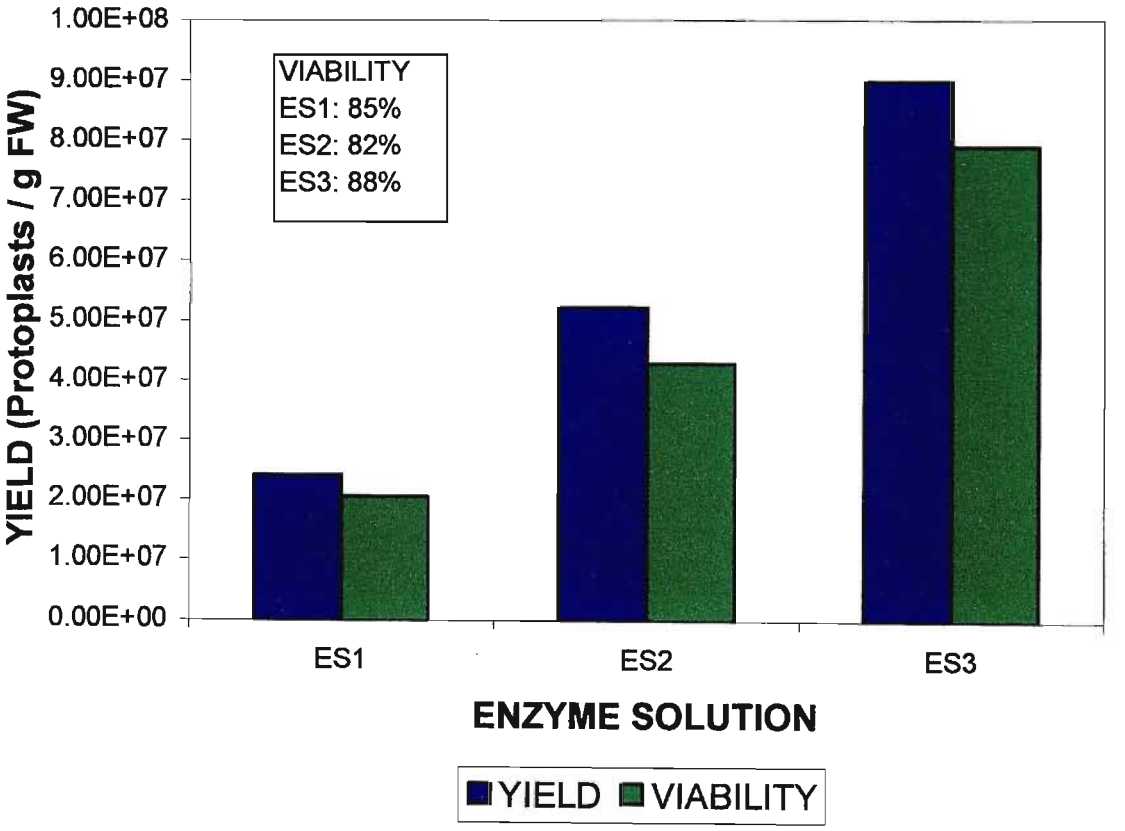


Figure 3.2

Yield and viability of protoplasts isolated from the mesocarp of a 9 month-old 'Hass' avocado using three enzyme solutions: ES1 (0.25% hemicellulase, 0.25% cellulase and 0.006% pectinase), ES2 (0.5% hemicellulase, 0.5% cellulase and 0.01% pectinase), and ES3 (1% hemicellulase, 1% cellulase and 0.02% pectinase)

According to Platt-Aloia *et al.* (1980) and Christoffersen *et al.* (1989), the mesocarp of avocado consists primarily of two cell types excluding vascular tissue. The majority of these cells are simple parenchyma cells containing multiple oil bodies (cell type 1) while the second cell type is termed an idioblast and is characterised by having a single large lipid body and secondary cell wall thickening. The protoplast suspension obtained from mesocarp tissue consisted of these cell types in approximately the same proportion as found in the source tissue (Figs. 3.3 a and b). In addition to these cells, however, a third cell type (cell type 3) was identified, containing between one and four oil droplets (Fig. 3.3 c). Protoplasts of idioblasts and cell type 3 were not observed and all cells had intact cell walls as shown by fluorescence of fluorochrome-bound (FB28) cellulose (Figs. 3.3 c (i) and (ii)). In early experiments, FB28 was dissolved in isolation media so that it could be incorporated into the culture media to monitor cell wall synthesis. This procedure, however, was discontinued when it was observed that type 1 cells, originally thought to be protoplasts because of their spherical shape, were not being stained properly and that fluorescent "cell wall debris" was in fact undissolved FB28. The existence of true protoplasts from mesocarp tissue was questioned and recent results suggest that cell wall digestion of the majority of type 1 cells was not complete and that, although single and completely detached, were, by definition, not real protoplasts. Fig. 3.4 clearly shows the presence of the cell wall around cells that were previously regarded as being protoplasts. So, were any protoplasts isolated? On further inspection of the suspension, several cells were identified which were spherical and showed no fluorescence when viewed under UV light (450-490 nm)(Fig. 3.5 a and b). The yield of these cells, however, was very low and it can be postulated that an anomaly in the isolation procedure or a component in the cells (e.g. lipids) prevented full digestion of the cell wall and subsequent release of the plasma membrane and its contents, known as the protoplast.

While the existence of protoplasts isolated from mesocarp tissue was later challenged, several procedures used for investigating membrane integrity and cell viability, were still of some value in contributing information about these cells and about the suitability of techniques. For instance, scanning electron

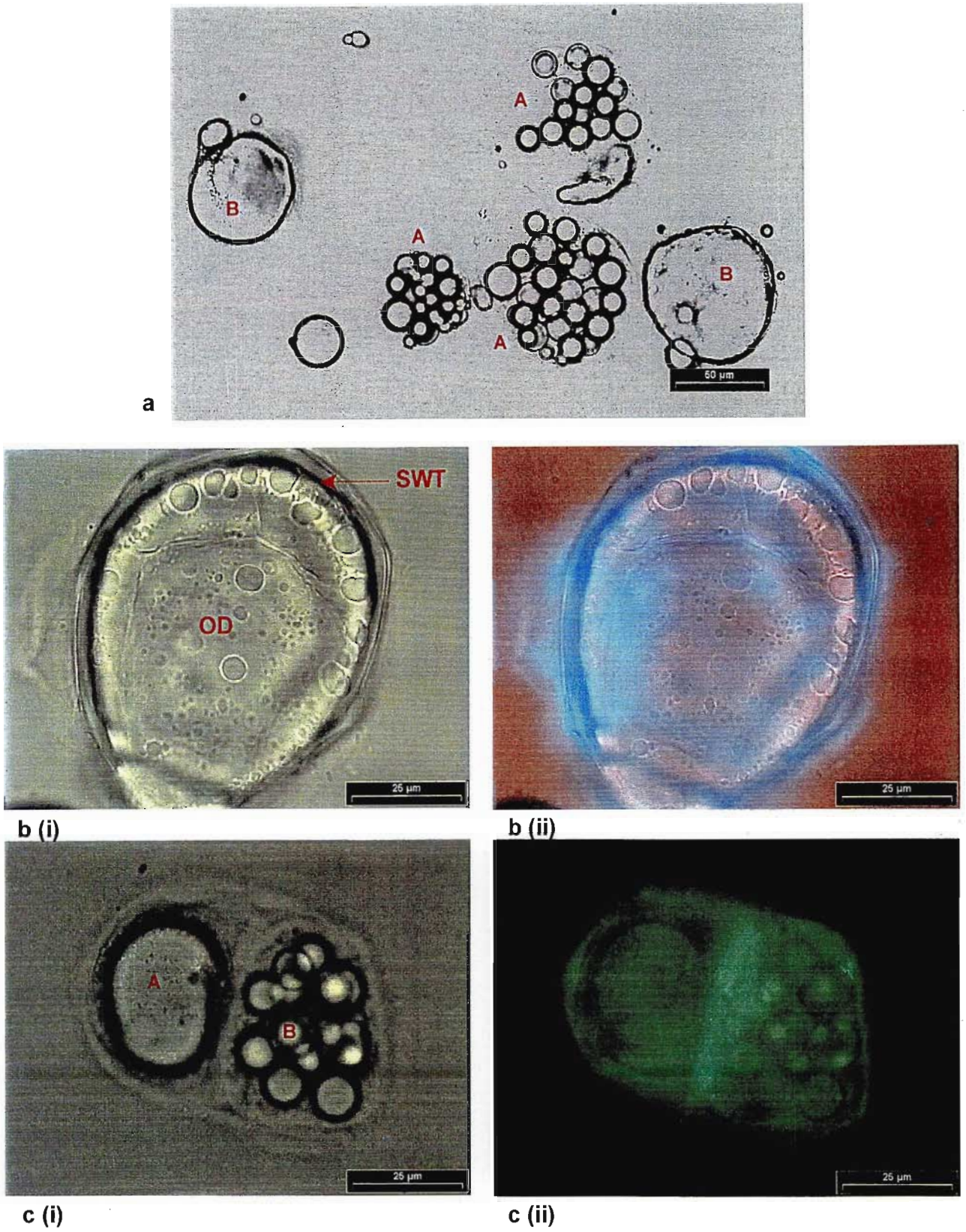
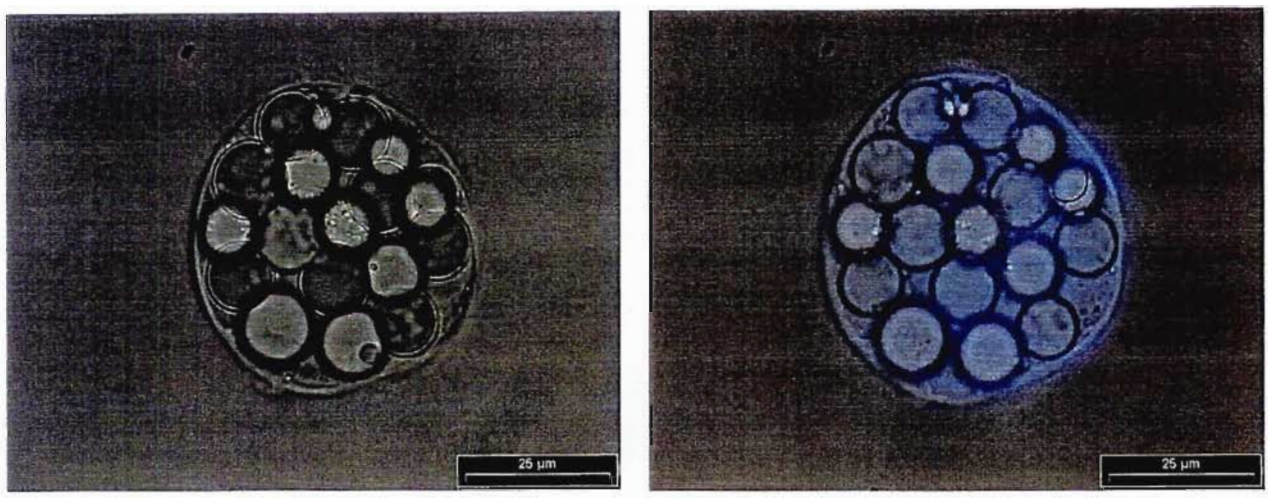


Figure 3.3

a. Micrograph showing the two cell types as described by Platt-Aloia *et al.* (1980) and Christoffersen *et al.* (1989) (A) Cells containing multiple oil bodies (B) Cells containing only one oil droplet.

b. Micrographs of an idioblast (cell type 2). i) Cell viewed under brightfield illumination. Note single oil droplet (OD) and secondary wall thickening (SWT). ii) Same cell viewed under UV (450-490 nm) after staining with FB28. Note fluorescence of intact cell wall.

c. i) Micrograph showing cell type 3 (A), with a single oil body, still attached to a type 1 cell (B) after overnight incubation in digestion medium. ii) Same cells viewed under UV following staining with FB28 showing intact cell walls



a b

Figure 3.4

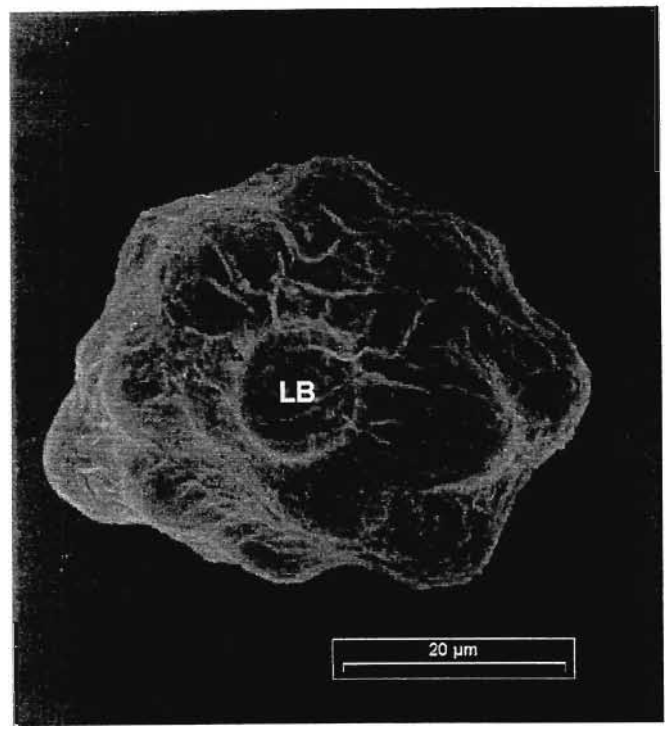
Micrographs of cells that were presumed to be protoplasts in early research. a) Spherical type 1 cell viewed under brightfield illumination b) Same field but viewed under UV (450-490 nm) following staining with 10 μgmL^{-1} FB28 dissolved in DMSO. Fluorescence indicates presence of cellulose and suggests that cell wall material still remains.

a b

Figure 3.5

Micrographs of protoplasts with completely digested cell walls. a) Single cell viewed under brightfield illumination b) Same cell viewed under UV (450-490 nm) following staining with 10 μgmL^{-1} FB28 dissolved in DMSO - the cell wall of an intact cell on the lower right (A) of the image fluoresces but no fluorescence is apparent from the cell in the centre, suggesting that complete digestion of the wall has taken place.

a



b

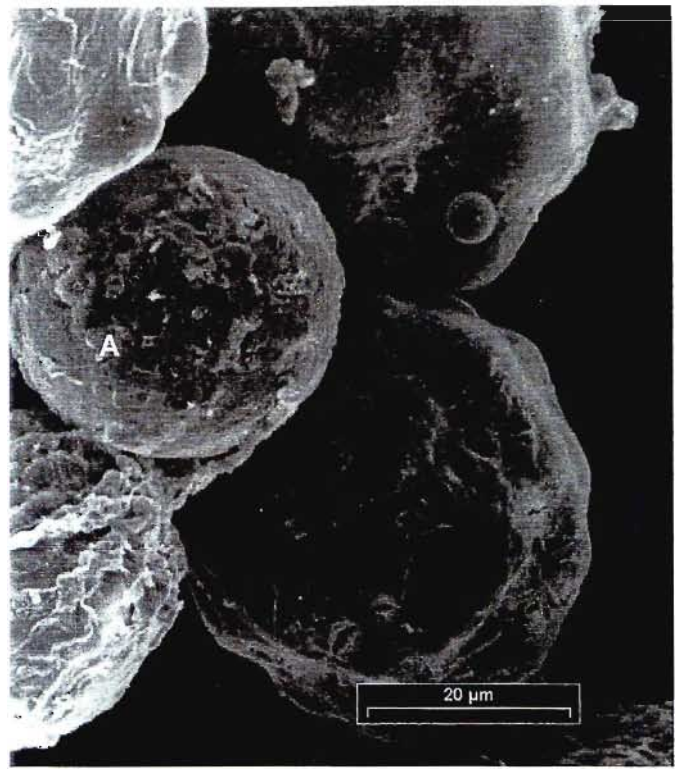


Figure 3.6

a) Scanning electron microscope image of a single type 1 cell. Lipid bodies are clearly visible by their shape but the opaque film of an incompletely digested cell wall prevents an inside view (cf Platt-Aloia *et al.* (1980) where a freeze-fracture micrograph of a ripe avocado fruit reveals a cell with no cell wall and a clear view of organelles inside).

b) Scanning electron micrograph of a group of cells including a type 3 cell (A) which is characterized by a smooth surface.

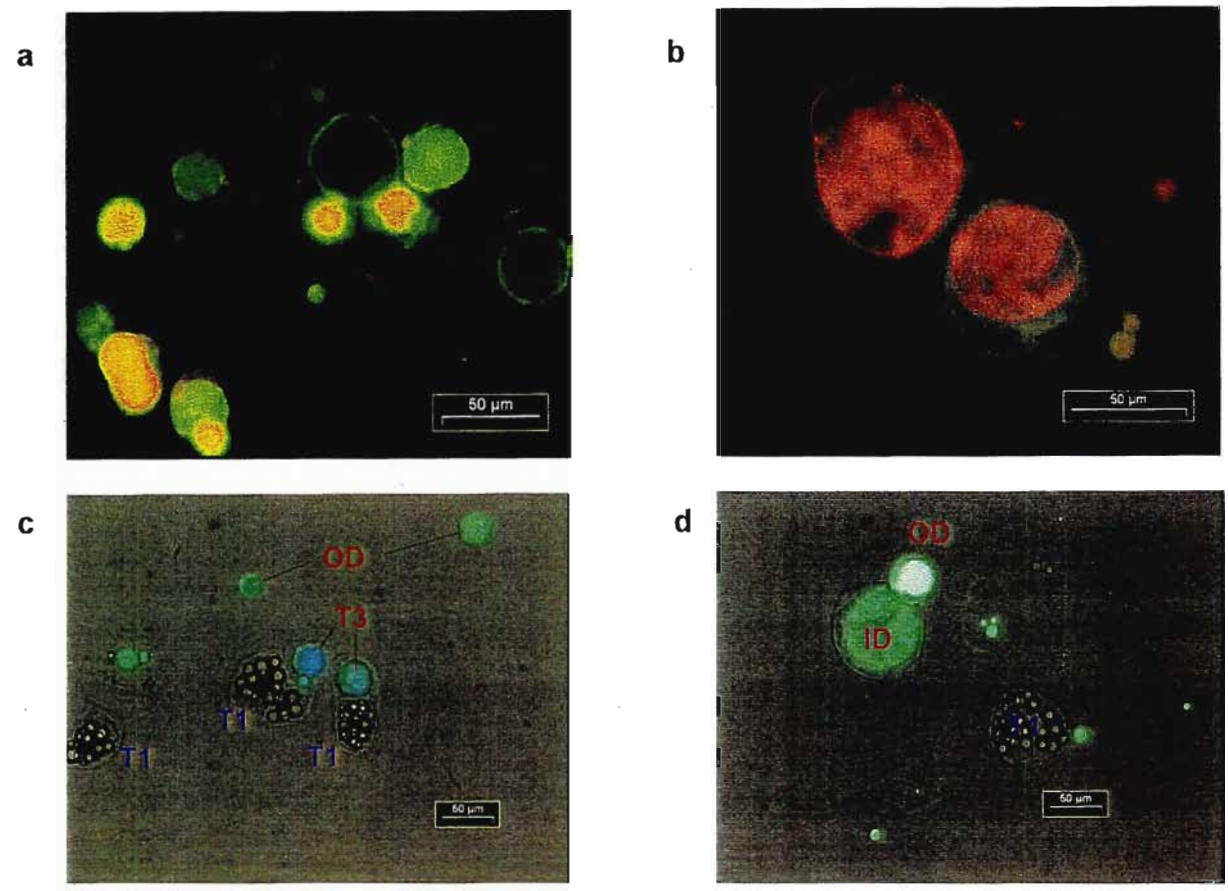


Figure 3.7

a and b) Micrographs showing type 3 cells stained with AO and viewed under UV (450-490 nm) after incubation in digestion medium. Viable cells are supposed to fluoresce green while non-viable cells fluoresce red. In the samples tested, however, there was no clear colour differentiation with many cells fluorescing orange. c and d) Micrographs showing freshly isolated cells stained with AO for < 5 min. Oil droplets (OD), idioblasts (ID) and type 3 cells (T3) fluoresce green while type 1 cells (T1) are not stained.

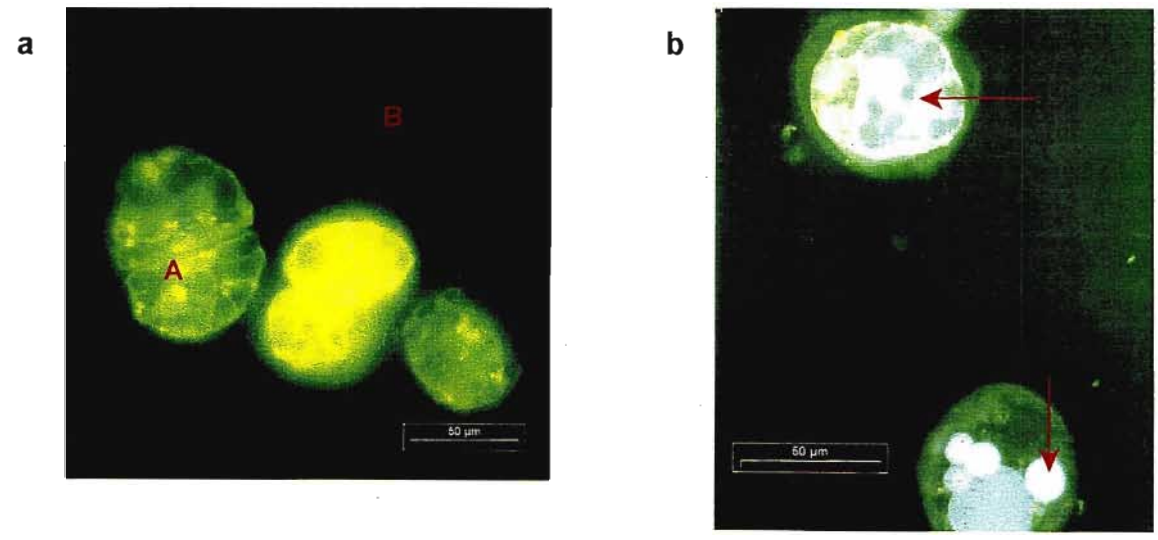


Figure 3.8

a) Micrograph of viable (A) and non-viable (B) type 1 cells stained with FDA and viewed under UV (450-490 nm).

b) Micrograph showing viable type 1 and type 3 cells stained with FDA. Note intense fluorescence in oil bodies compared to cytoplasmic fluorescence.

microscope (SEM) images confirmed the presence of at least two cell types (type 1, Fig. 3.6a and either an idioblast or type 3 cell, Fig. 3.6b). The multiple lipid bodies in type 1 cells were clearly visible (Figs. 3.6a and b) but the non-spherical shape and opaque film suggests that residual cell wall material remained.

The use of vital stains for viability testing of presumed protoplasts yielded interesting data on the use of these techniques. The three dyes used included phenosafranin, an exclusion dye, acridine orange (AO) and flourescein diacetate (FDA), both of which are vital fluorochrome dyes. Phenosafranin proved to be the most consistent in terms of variation of results but was still subjective especially when used in semi-purified (filtered but unwashed) preparations where background staining of damaged cells confounded interpretation. The fluorochrome dyes, AO and FDA were highly subjective and very time dependent. Dead and living cells stained with AO are supposed to fluoresce red and green respectively. The colour of fluorescence in the suspensions tested, however, ranged from yellowish-green to orange through all shades of the two extremes (Figs. 3.7a and b). In addition, time of incubation was critical with very short periods (<5 min) resulting in the staining of idioblasts, type 3 cells and oil droplets only (Fig. 3.7 c and d). FDA was less difficult to interpret since viable cells fluoresced and non-viable cells did not but staining time was critical. After approximately 10-15 min after staining, non-specific, diffuse fluorescence began to appear in most cells and the culture medium, obscuring interpretation. Viable and non-viable cells are shown in Figs. 3.8a and b.

3.2.2 Callus induction

3.2.2.1 Mesocarp explants

The induction of callus tissue from mesocarp explants of 7 – 8 month-old fruit was slow to begin. After five weeks of culture, four treatments (see Fig. 3.9) gave reasonable results – NAA / BA (5 mgL^{-1} / 1 mgL^{-1}); NAA / iP (5 mgL^{-1} / 0.5 mgL^{-1}); NAA / iP (5 mgL^{-1} / 1 mgL^{-1}) and IBA / iP (5 mgL^{-1} / 0.5 mgL^{-1})(Fig. 3.10 a to d). By week three, many of the mesocarp sections had turned black,

and, by week five, had become totally desiccated and split open (Fig. 3.10 e). Callus forming on explants that did survive, usually originated at the interface of the media and explant and had a hard, lumpy, wet consistency.

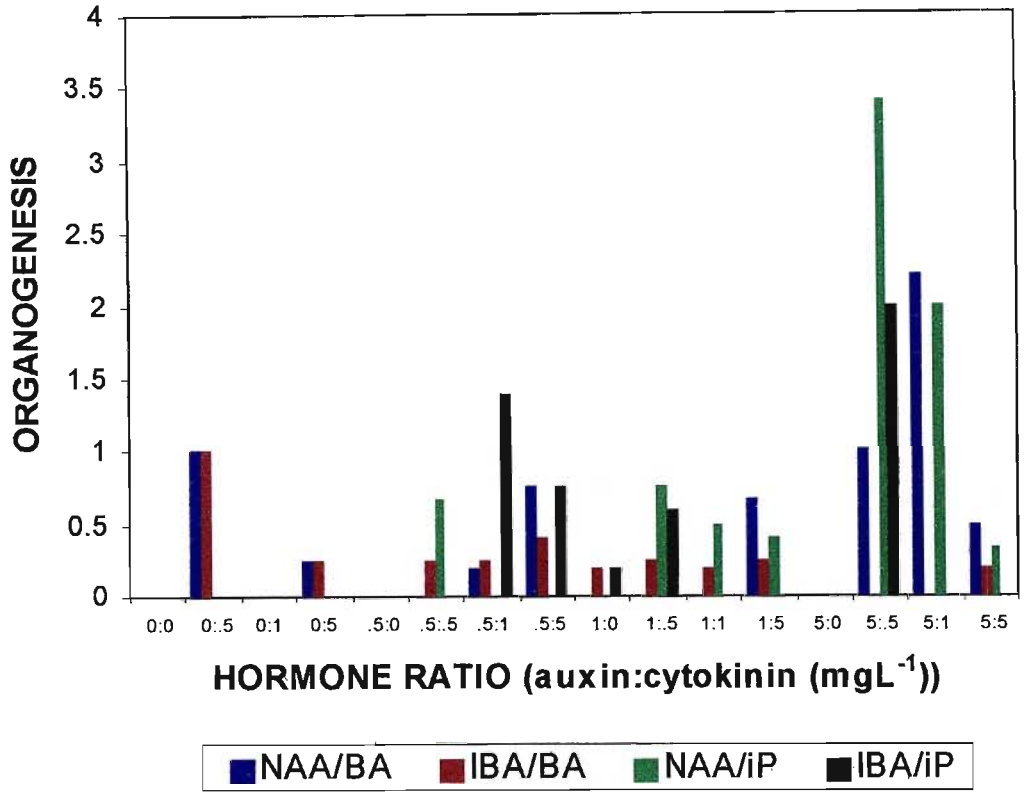


Figure 3.9

Organogenesis from mesocarp explants after 5 weeks of culture. Values are means of 5 replications where growth was measured using an arbitrary scale of 0 to 10 where 0 = no callus, 1 = the appearance of callus, 5 = half of the explant covered in callus and 10 = no explant visible (see section 2.13.2)

Organogenesis from mesocarp-derived callus was never observed although bud-like structures did develop after prolonged culture on media with a high AUX (IBA):CK (iP) ratio. Interestingly, the absence of either of these plant growth regulators resulted in very poor growth and in most cases, tissue blackening and necrosis. The combination of IBA and BA at all concentrations elicited little response while low concentrations (<5 mgL^{-1}) of either IBA or NAA resulted in almost no proliferation (Fig 3.9). Best results in terms of callus induction and growth, were obtained at high AUX concentrations (5 mgL^{-1}) and relatively low CK levels (0.5-1 mgL^{-1}). Subsequent testing of elevated

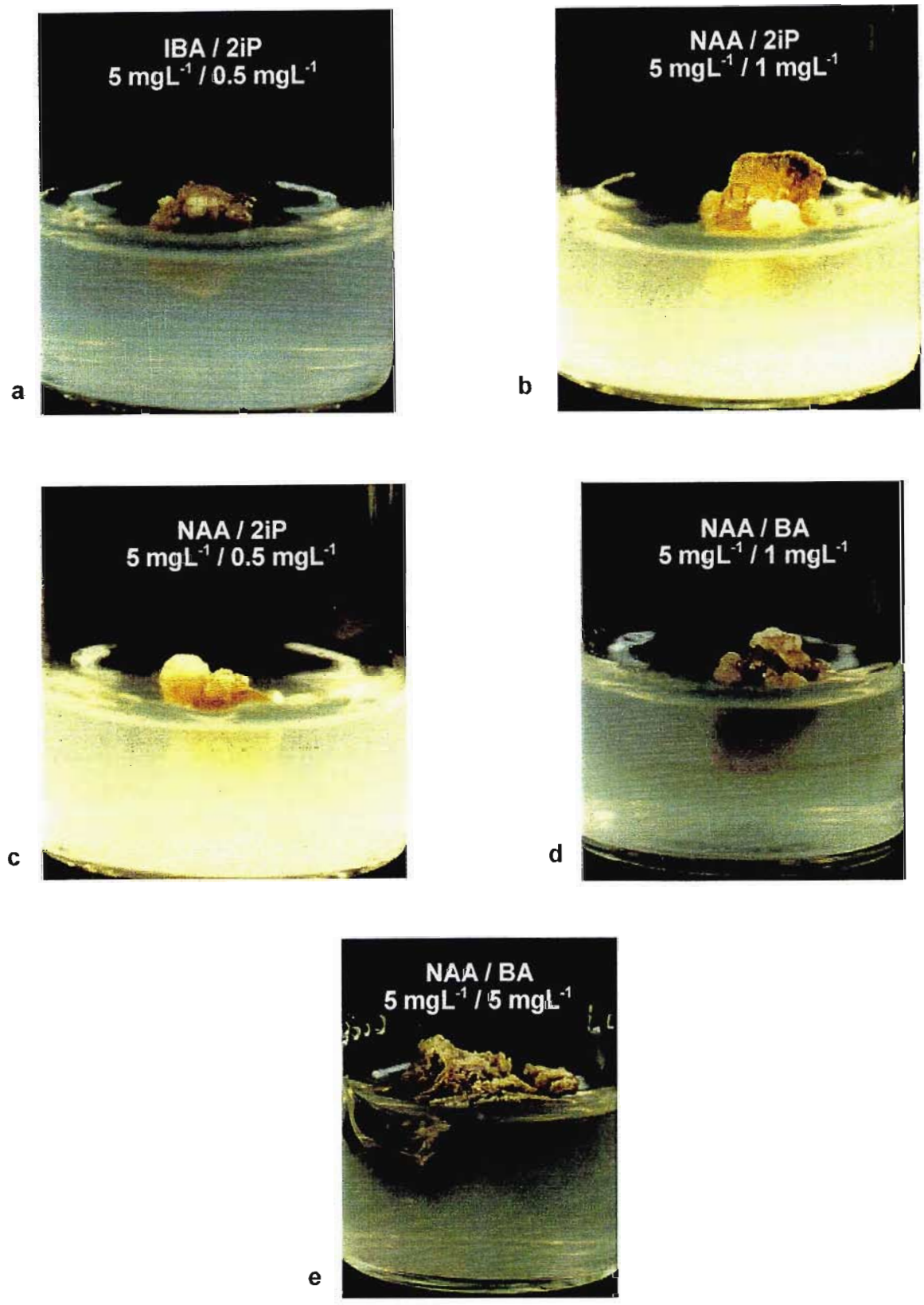


Figure 3.10

Callus induction from mesocarp tissue after 5 weeks of culture. a) to d) are representative examples of the four best treatments. e) shows a mesocarp explant at 5 weeks with excessive tissue blackening.

AUX levels (10 and 20 mgL^{-1}) (data not shown) proved to be ineffective in promoting more, or faster cell dedifferentiation, and in most cases, explants blackened and died.

3.2.2.2 Seed explants

Callus tissue was induced to grow readily from seed tissue of 7 - 8 month-old 'Hass' avocados. High concentrations of growth regulators, in particular NAA / iP (5 mgL^{-1} / 5 mgL^{-1}) and IBA / iP (5 mgL^{-1} / 5 mgL^{-1}) resulted in fastest callus induction and growth (Fig. 3.11).

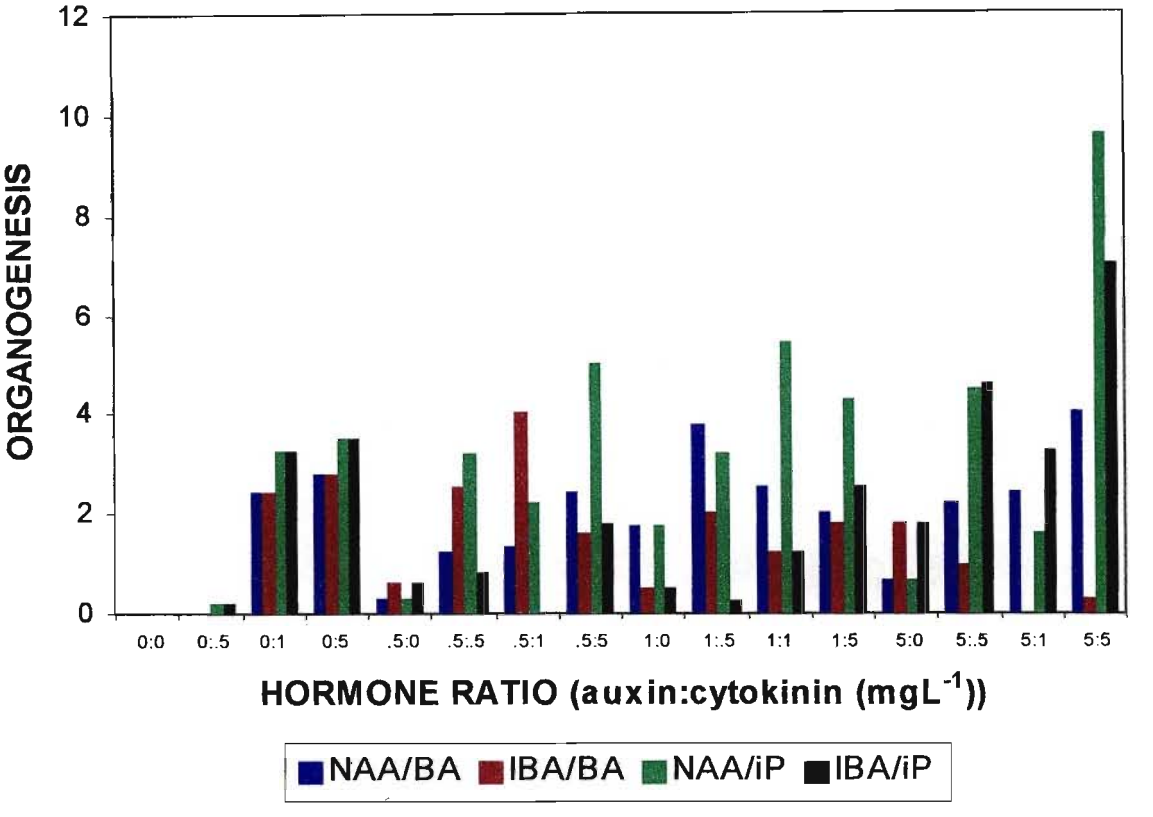


Figure 3.11

Organogenesis from seed explants after 5 weeks of culture. Values are means of 5 replications where growth was measured using an arbitrary scale of 0 to 10 where 0 = no callus, 1 = the appearance of callus, 5 = half of the explant covered in callus and 10 = no explant visible (see section 2.13.2)

The outer layers of cells in endosperm explants discoloured rapidly through oxidation to give a rust-coloured surface to the sections. Callus generally

proliferated from most explants but it was observed that severely oxidised tissue either produced no callus and slowly necrotized, or produced callus after a long incubation period which was neither fast growing nor long-lived. Sterilization of seed tissue explants by soaking in commercial bleach for any length of time resulted in severe oxidation and an almost 100% cull rate of planted tissue.

Callus derived from seed tissue was phenotypically less homogenous than that from mesocarp tissue as can be seen by comparing Fig 3.10 a – e with Fig. 3.12 a – e. Morphologically different callus appeared from endosperm and nucellar tissue. Hard, white, crystalline callus originated mainly from endosperm tissue (Fig 3.12 a) while wet, lumpy, friable callus was induced from nucellar tissue (Fig. 3.12 b). At high hormone concentrations, however, both types were visible on many explants (Fig. 3.12 c). In addition to these types, some explants on media with a high AUX:CK ratio, developed nodular structures (Fig. 3.12 d) which neither differentiated further nor proliferated into callus tissue. A further callus type was identified which was very light, friable and seemingly dry (Fig. 3.12 e). This callus type, which was frequently found developing from the peripheral cells of the seed, would often be the first callus to become necrotic. Subculture of this callus onto fresh solid media resulted in rapid browning (after 2–3 d) but subculturing into liquid media resulted in the formation of globular callus structures within 5-7 d. Subsequent manipulations of these structures failed to induce differentiation into an organised, recognisable form. The wet, lumpy callus proved to be the most amenable to subculture and rapid cell division was observed up to six weeks after transfer. Homogeneic callus cultures were never observed – wet, lumpy callus explants (which were used exclusively for subculture) produced hard, nodular callus and, after prolonged culture without change in media, the light, friable dry callus type. Culture under continuous light and in media with a high AUX:CK ratio (particularly IBA:iP) resulted in the formation of callus with individual globular structures seemingly independent of vascular connection to the mother tissue. Thought to be somatic embryos in the globular stage of development, these were transferred to various media (see Table 3.1) in an

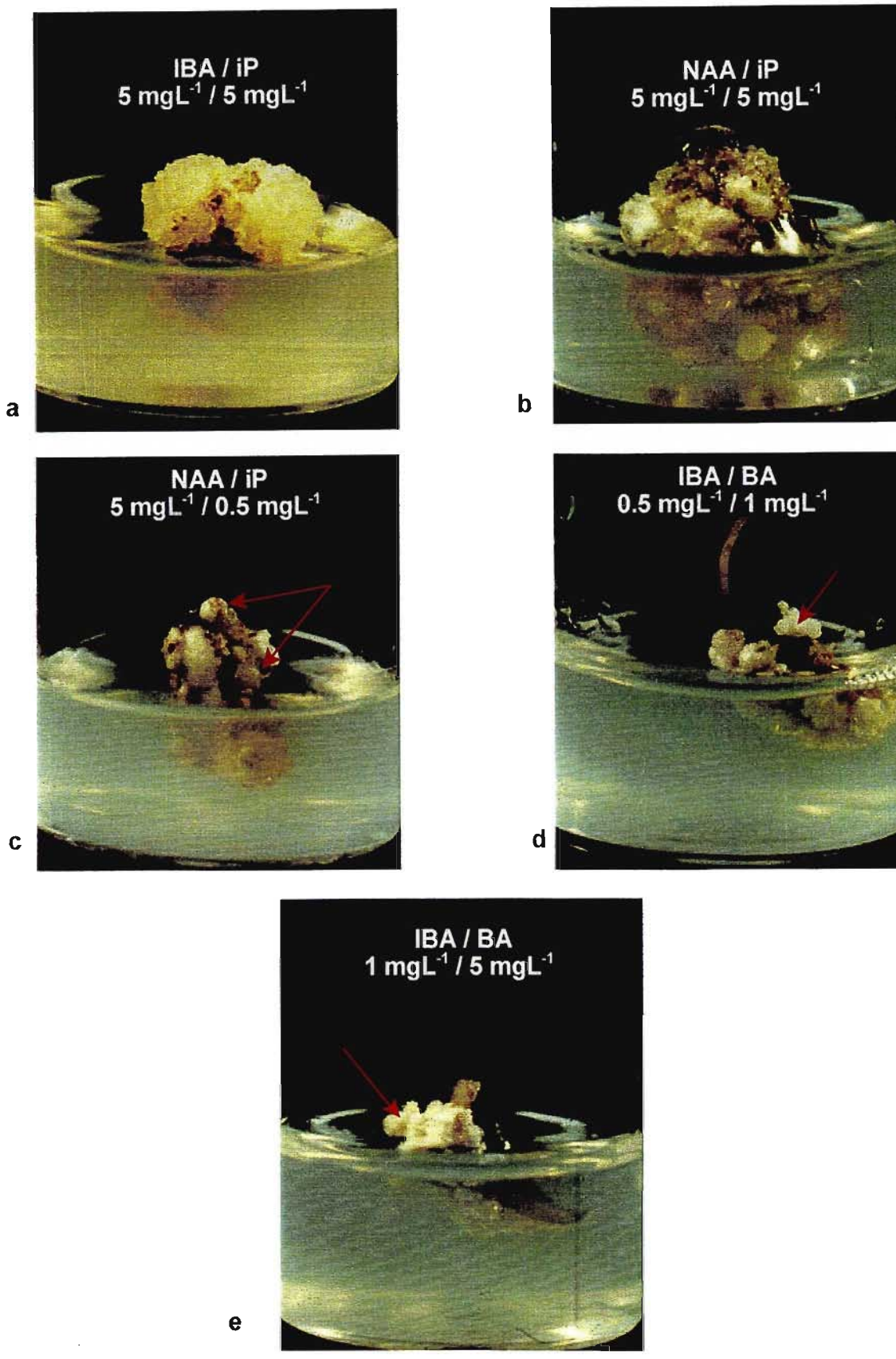


Figure 3.12

Callus induction from seed tissue. a) Wet, lumpy callus which responded well to subculture to solid and liquid media, originated mainly from nucellar tissue. b) Heterogenic callus mass including all callus types. c) Nodular structures produced from seed explant at high AUX:CK ratios. d) Hard, white, crystalline callus originating mainly from endosperm tissue. e) Light, friable, dry callus tissue produced from the periphery of seed tissue.

attempt to induce further differentiation. After 6 weeks of culture, however, the structures blackened and became necrotic.

Table 3.1

Treatments applied to nodular structures, thought to be somatic embryos, to try and induce further development

Media	Treatment	Result
Solid MSB	No hormones, 400 mgL^{-1} glutamic acid, 0.1% activated charcoal	/
Solid ½ MSB	No hormones, 400 mgL^{-1} glutamic acid, 0.1% activated charcoal	/
Solid B5	No hormones, 400 mgL^{-1} glutamic acid, 0.1% activated charcoal	/
Solid MSB	NAA (0.2 mgL^{-1}), 400 mgL^{-1} glutamic acid, 0.1% activated charcoal	/
Solid MSB	Picloram (0.1 mgL^{-1}), 0.05% activated charcoal	/
Solid ½ MSB	NAA (0.2 mgL^{-1}), 400 mgL^{-1} glutamic acid, 0.1% activated charcoal	/
Solid B5	NAA (0.2 mgL^{-1}), 400 mgL^{-1} glutamic acid, 0.1% activated charcoal	/
Solid Dixon and Fuller (1976)	No hormones	/
Liquid MSB	No hormones, 400 mgL^{-1} glutamic acid	/
Liquid ½ MSB	No hormones, 400 mgL^{-1} glutamic acid	/
Liquid B5	No hormones, 400 mgL^{-1} glutamic acid	/
Liquid MSB	NAA (0.2 mgL^{-1}), 400 mgL^{-1} glutamic acid	/
Liquid ½ MSB	NAA (0.2 mgL^{-1}), 400 mgL^{-1} glutamic acid	/
Liquid B5	NAA (0.2 mgL^{-1}), 400 mgL^{-1} glutamic acid	/

Organogenesis and rhizogenesis from seed explants was occasionally observed (Fig. 3.14 a and b) although the origin of the organ in all cases was obscured. Both roots and shoots developed from swollen nodes (Fig. 3.14 a) or callus masses (Fig. 3.14 b) close to the original explant giving rise to speculation that embryogenic tissue was still attached to the section. Indirect rhizogenesis was observed only once from subcultured callus tissue growing on Woody Plant Medium (WPM)(Lloyd and McCowan, 1981) supplemented with NAA (0.5 mgL^{-1}) and thidiazuron (TDZ) (0.05 mgL^{-1}) (data not shown). Neither shoots nor roots, produced by direct or indirect organogenesis survived while still attached to the mother tissue.

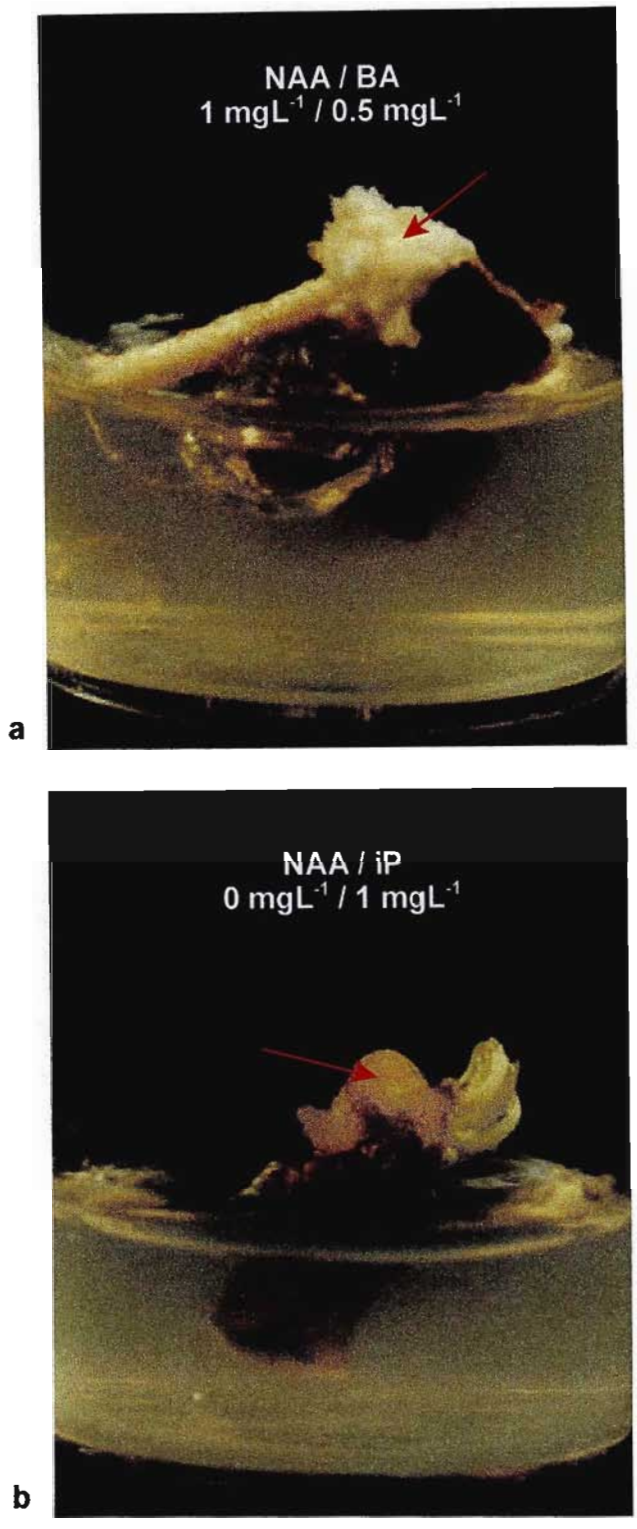
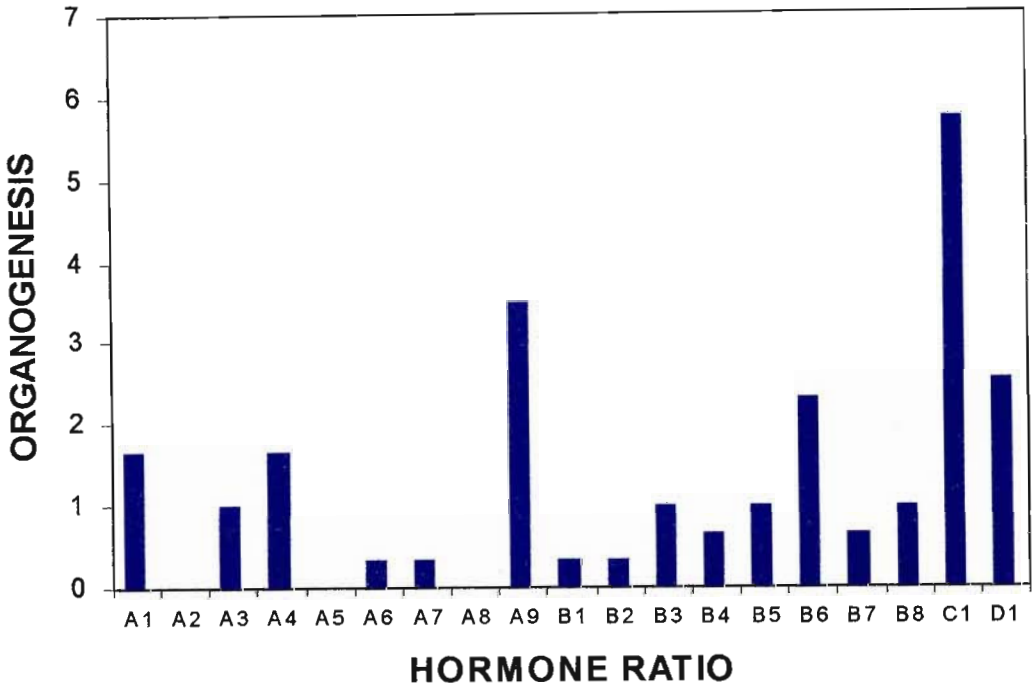


Figure 3.14

Organogenesis and rhizogenesis from seed explants. a) Root formation from callus tissue b) Shoot formation from swollen nodes. It was speculated that, because the explant was still attached, the origin of these structures was within the parent tissue resulting in this being classified as direct organogenesis from predetermined meristemmatic cells as opposed to indirect organogenesis from induced shoot / root meristemmatic cells.

3.2.2.3 Embryo explants

Callus tissue was successfully induced to grow from cultured embryos, excised from 8 – 9 month-old fruit, by high AUX:CK treatments particularly NAA / iP (5 mgL^{-1} / 0.5 mgL^{-1}) and IBA / iP (5 mgL^{-1} / 0.5 mgL^{-1})(Fig. 3.15).



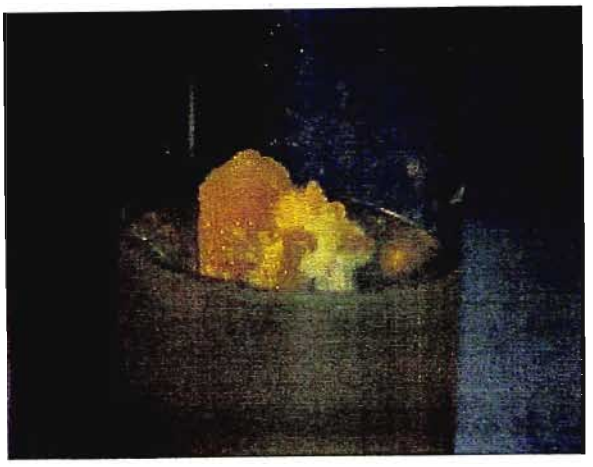
HORMONE RATIOS (auxin:cytokinin (mgL^{-1}))	
A1 – IBA:iP (0.3:0.01)	B1 – IBA:Kn (0.3:0.01)
A2 – IBA:iP (0.3:0.1)	B2 – IBA:Kn (0.3:0.1)
A3 – IBA:iP (0.3:0.3)	B3 – IBA:Kn (0.3:0.3)
A4 – IBA:iP (0.3:1)	B4 – IBA:Kn (0.3:1)
A5 – IBA:iP (1:0.01)	B5 – IBA:Kn (1:0.01)
A6 – IBA:iP (1:0.1)	B6 – IBA:Kn (1:0.1)
A7 – IBA:iP (1:0.3)	B7 – IBA:Kn (1:0.3)
A8 – IBA:iP (1:1)	B8 – IBA:Kn (1:1)
A9 – IBA:iP (5:0.5)	C1 – NAA:iP (5:0.5)
	D1 – 2, 4-D:iP (5:0.5)

Figure 3.15

Organogenesis from excised embryos after 5 weeks of culture. Values are means of 5 replications where growth was measured using an arbitrary scale of 0 to 10 where 0 = no callus, 1 = the appearance of callus, 5 = half of the explant covered in callus and 10 = no explant visible (see section 2.13.2)

Callus produced was almost exclusively wet and lumpy with a yellowish colour (Fig. 3.16 a – c). It responded well to subculture onto media of the same

a

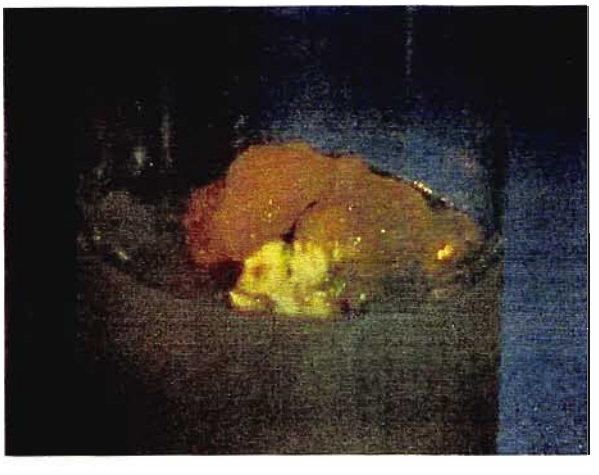


2.4D / iP
(5 mgL^{-1} / 0.5 mgL^{-1})

b

NAA / iP
(5 mgL^{-1} / 0.5 mgL^{-1})

c



IBA / iP
(5 mgL^{-1} / 0.5 mgL^{-1})

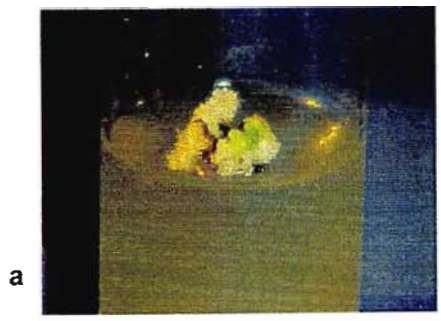
Figure 3.16

Callus induction from excised 'Hass' avocado embryos from 8 - 9 month-old fruit. a to c) A high AUX : CK ratio was most effective in inducing callus to grow. The callus produced was almost exclusively wet and lumpy with a yellowish colour that proved to be very amenable to subculture into liquid media or onto solid media.

composition as that from which it was induced, although the addition of activated charcoal (0.5 gL^{-1}) seemed to promote growth. The other plant growth regulator concentrations were based on the results of Gonzalez-Rosas *et al.* (1990) who used various combinations of IBA, Kn and BA to investigate germination responses, and root and shoot development of excised *P. americana* var *americana* R. Mexicana embryos. Their results indicated that the percentage germination and number of roots and shoots was highest when embryos were cultured in MS medium supplemented with IBA / Kn (0.3 mgL^{-1} / 1 mgL^{-1}). Further, they observed that the presence of BA depressed shoot growth after germination. The results shown in Fig. 3.17 a – p displayed no positive trends for any of the treatments used. The combination of IBA / iP resulted in general greening of most explants but after five weeks of culture, no germination *per se* had occurred. IBA / Kn treatments also did not evoke any uniform responses – two treatments, IBA / Kn (0.3 mgL^{-1} / 0.1 mgL^{-1}) and IBA / Kn (0.3 mgL^{-1} / 0.3 mgL^{-1}) resulted in the production of a single root and a single shoot respectively. Total germination, with the production of a root and a shoot from the same explant, was never observed. While several other embryos in different treatments produced shoots after prolonged culture (7 –8 months), it was observed that all shoots experienced shoot tip die back resulting in eventual death of the whole explant. This phenomenon was noted even when the explant was subcultured onto fresh media in larger culture vessels.

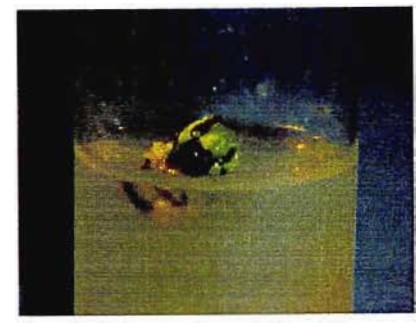
3.2.3 Initiation and maintenance of cell suspension cultures

Liquid cell cultures were successfully started through the transfer of wet, lumpy callus derived from nucellar tissue, to nutrient media. Initially, callus tissue was cultured in liquid media with the same hormone composition as the solid media from which the callus was induced / maintained. After four weeks, however, it became apparent that the combination of NAA / iP (5 mgL^{-1} / 1 mgL^{-1}) promoted faster growth and all cell cultures were incubated in this medium until further testing could be carried out. It was observed that neither the hard, white, crystalline callus nor the light, friable dry callus type formed cell suspensions.



a

IBA / iP
(0.3 mgL^{-1} / 0.01 mgL^{-1})



e

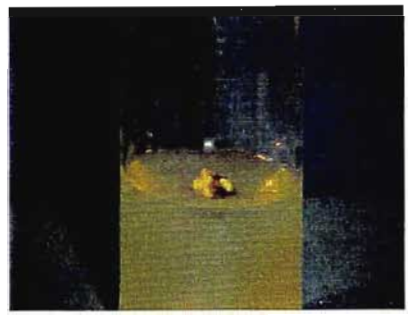
IBA / iP
(1 mgL^{-1} / 0.01 mgL^{-1})

b

IBA / iP
(0.3 mgL^{-1} / 0.1 mgL^{-1})

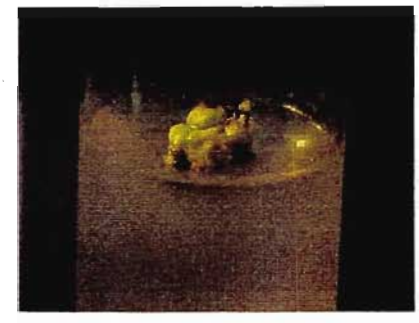
f

IBA / iP
(1 mgL^{-1} / 0.1 mgL^{-1})



c

IBA / iP
(0.3 mgL^{-1} / 0.3 mgL^{-1})

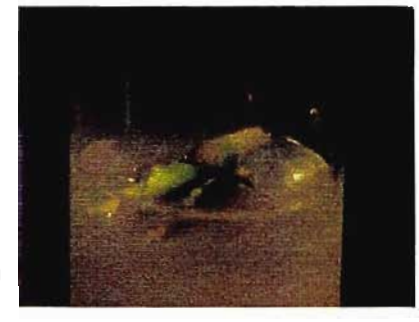


g

IBA / iP
(1 mgL^{-1} / 0.3 mgL^{-1})

d

IBA / iP
(0.3 mgL^{-1} / 1 mgL^{-1})

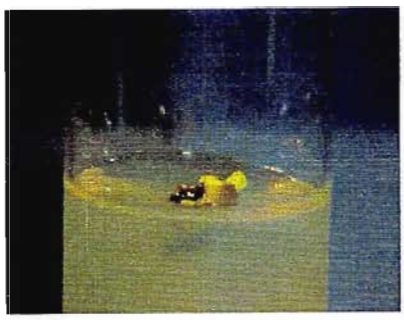


h

IBA / iP
(1 mgL^{-1} / 1 mgL^{-1})

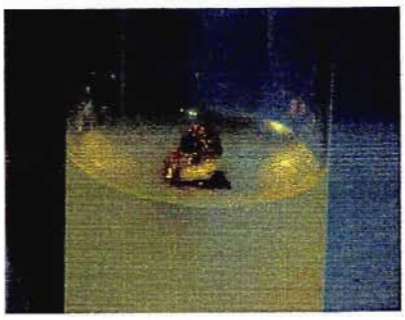
Figure 3.17

Culture of excised 8 - 9 month-old 'Hass' avocado embryos. a -h) IBA / iP treatments resulted in general greening of the explants after 5 weeks of culture but no germination was observed.



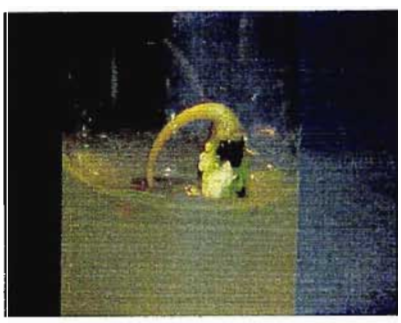
i

IBA / Kn
(0.3 mgL^{-1} / 0.01 mgL^{-1})



m

IBA / Kn
(1 mgL^{-1} / 0.01 mgL^{-1})



j

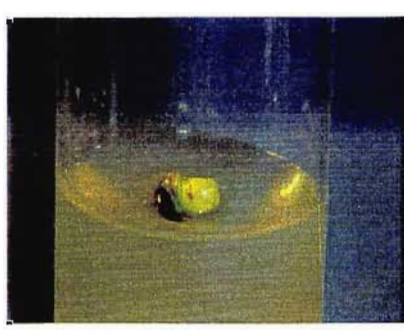
IBA / Kn
(0.3 mgL^{-1} / 0.1 mgL^{-1})

n

IBA / Kn
(1 mgL^{-1} / 0.1 mgL^{-1})

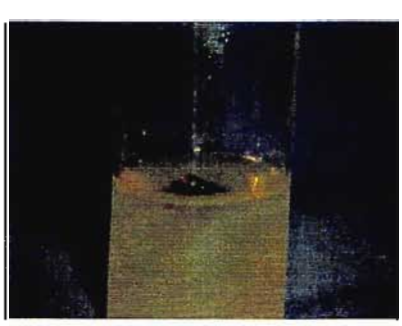
k

IBA / Kn
(0.3 mgL^{-1} / 0.3 mgL^{-1})



o

IBA / Kn
(1 mgL^{-1} / 0.3 mgL^{-1})



l

IBA / Kn
(0.3 mgL^{-1} / 1 mgL^{-1})

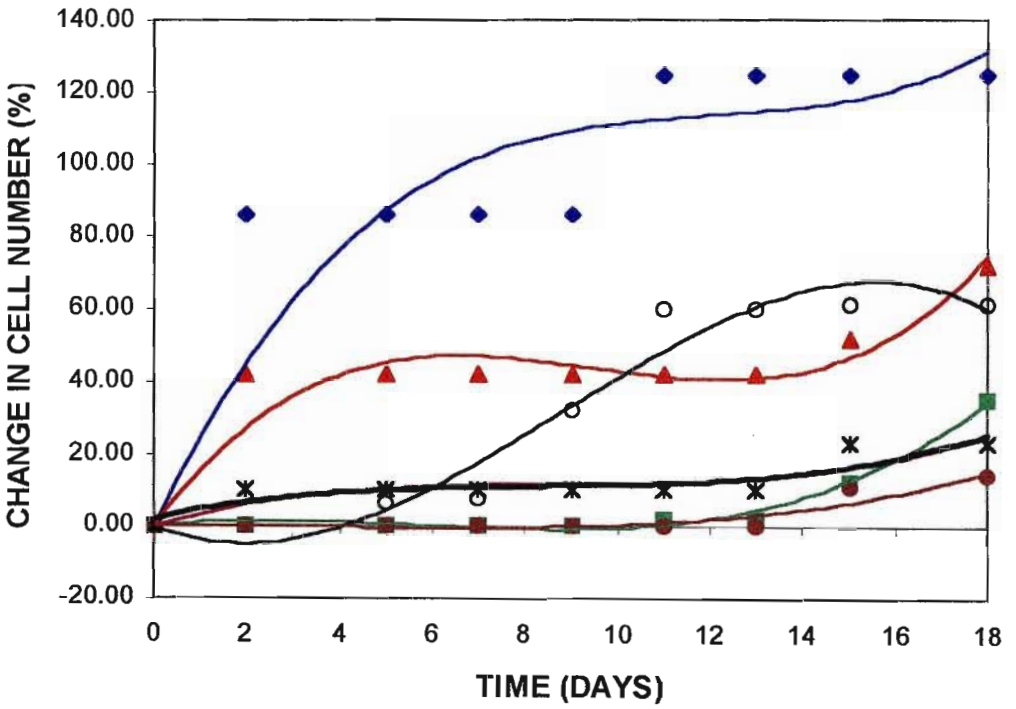
p

IBA / Kn
(1 mgL^{-1} / 1 mgL^{-1})

Figure 3.17

Culture of excised 8 - 9 month-old 'Hass' avocado embryos. i - p) IBA / Kn treatments gave mixed results with two of the treatments (0.3 mgL^{-1} / 0.1 mgL^{-1} and 0.3 mgL^{-1} / 0.1 mgL^{-1}) giving rise to a root and a shoot respectively, although this was not reflective of all replications in these treatments.

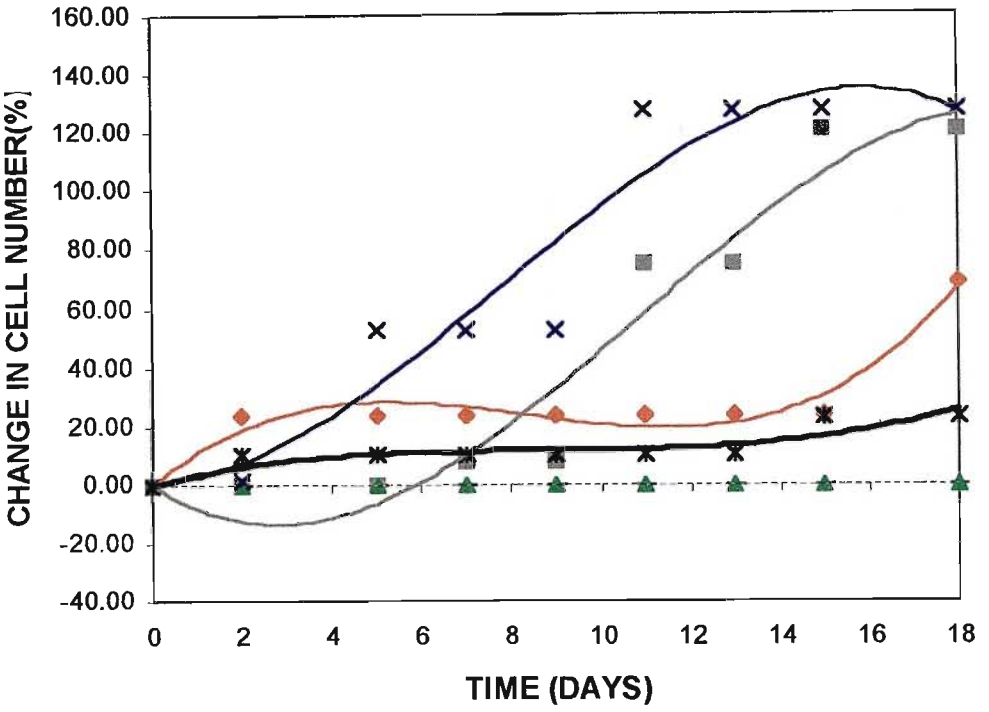
Media testing for optimal growth included two auxins, 2, 4-D and NAA, and the cytokinin iP. Cell population growth was estimated by determining cell number in a fixed volume of suspension. The results are illustrated in Fig. 18 a and b.



◆ 2, 4-D 0.2 mgL^{-1}	$y = 0.0593x^3 - 2.1392x^2 + 26.581x$, $r^2 = 0.7819$
■ 2, 4-D 0.5 mgL^{-1}	$y = 0.0217x^3 - 0.3613x^2 + 1.392x$, $r^2 = 0.9878$
▲ 2, 4-D 1 mgL^{-1}	$y = 0.0718x^3 - 2.0292x^2 + 17.382x$, $r^2 = 0.8941$
○ 2, 4-D 5 mgL^{-1}	$y = 0.0573x^3 + 1.5056x^2 - 5.2153x$, $r^2 = 0.9558$
● 2, 4-D / 2iP 5 mgL^{-1} / 1 mgL^{-1}	$y = 0.0062x^3 - 2.1392x^2 + 26.581x$, $r^2 = 0.8797$
x CONTROL	$y = 0.0163x^3 - 0.4237x^2 + 3.7864x$, $r^2 = 0.7901$

Figure 3.18a

Growth curves of cells cultured in MSB media containing different concentrations of 2, 4-D and iP. Data were collected from two replicates of each treatment and the experiment was duplicated. Fitted curves are 3 order polynomials (sigmoidal) with a zero intercept created by the statistical analysis function of Microsoft Excel®.

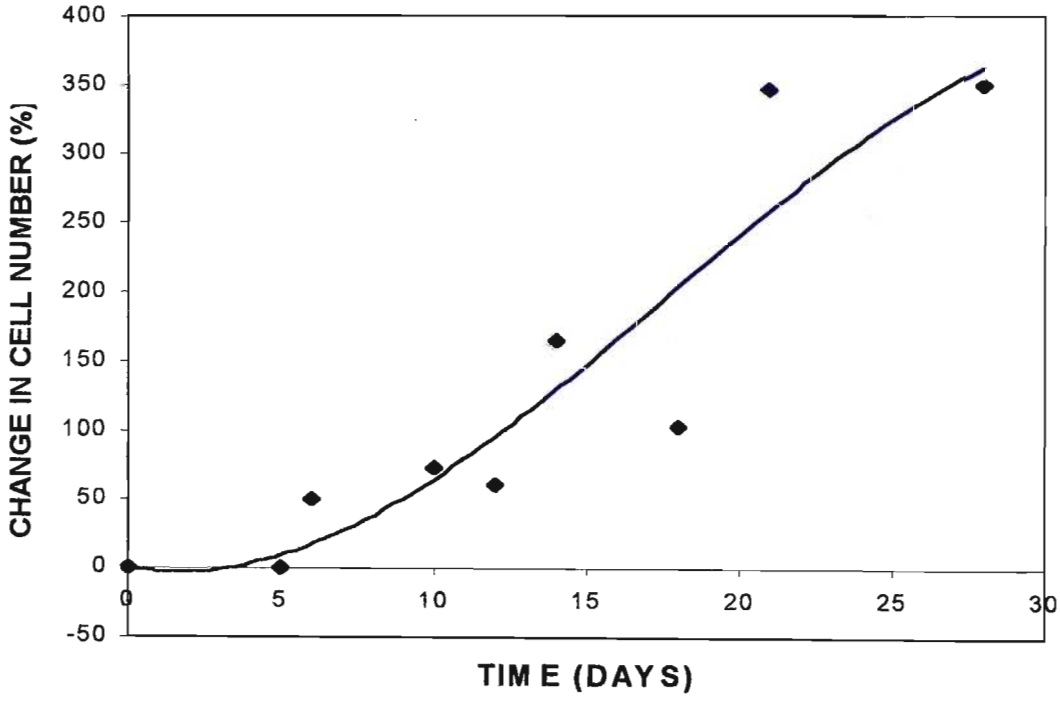


◆ NAA 0.2 mgL^{-1}	$y = 0.0706x^3 - 1.7727x^2 + 12.734x$ $r^2 = 0.9504$
■ NAA 0.5 mgL^{-1}	$y = 0.0653x^3 + 2.1352x^2 - 10.305x$ $r^2 = 0.9878$
▲ NAA 1 mgL^{-1}	N/A
○ NAA / 2iP 5 mgL^{-1} / 1 mgL^{-1}	$y = 0.063x^3 + 1.4692x^2 + 1.0154x$ $r^2 = 0.9221$
x CONTROL	$y = 0.0163x^3 - 0.4237x^2 + 3.7864x$ $r^2 = 0.7901$

Figure 3.18b

Growth curves of cells cultured in MSB media containing different concentrations of NAA and iP. Data were collected from two replicates of each treatment and the experiment was duplicated. Fitted curves are 3 order polynomials (sigmoidal) with a zero intercept created by the statistical analysis function of Microsoft Excel®.

While no trends, with respect to response to changes in hormone levels, were apparent, three treatments resulted in a doubling of cell population (2, 4-D 0.2 mgL^{-1}; NAA 1 mgL^{-1}; NAA / iP 5 mgL^{-1} / 1 mgL^{-1}). Of these, NAA / iP (5 mgL^{-1} / 1 mgL^{-1}) produced the most consistent results in two separate experiments and was chosen for all subsequent cell studies. Freshly transferred callus tissue took approximately 10 d to acclimatize to liquid media and by day 14, a noticeable cell division "boom" had occurred. In subsequent subcultures, large aggregates were excluded from transfer by using a thin bored pipette to remove culture solution. Growth of these single cells and small aggregates followed a similar pattern to that in Fig. 3.18 with a lag phase of approximately 10 d and an exponential phase of 12 to 15 d (Fig. 3.19).



$y = 0.0247x^2 + 1.3007x^2 - 4.0875x$
$r^2 = 0.85$

Figure 3.19

Population dynamics of cells derived from 'Hass' avocado seed callus tissue incubated in MSB liquid media containing NAA / iP (5 mgL^{-1} / 1 mgL^{-1}). Data were collected from 9 replicates. The fitted curve is a 3 order polynomial (sigmoidal) with a zero intercept created by the statistical analysis function of Microsoft Excel®.

Cells were generally oval to rectangular in shape (Fig. 3.20 a to c) although thin, cylindrical as well as large irregularly shaped cells were not uncommon.

3.2.4 Culture of mesocarp protoplasts

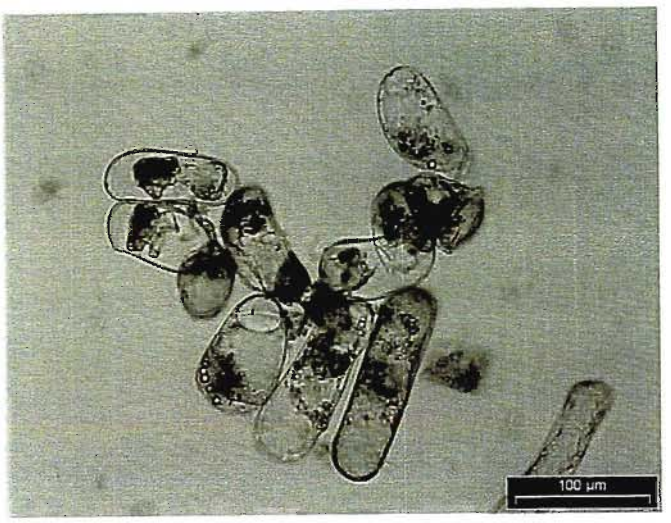
Culture of protoplasts isolated from mesocarp tissue was attempted to test their viability and totipotency as well as to establish solid and liquid cultures from single cells. The methods employed were based on those of Dhir *et al.* (1991) who used liquid culture and agarose-containing media to culture protoplasts of soybean (*Glycine max* L.) cotyledons at low population densities.

After seven weeks in culture, none of the cells in the four culture methods had proliferated. Cell wall synthesis (Fig. 3.21 a and b), monitored by the inclusion of fluorescent brightener 28 (10 μgmL^{-1}), was originally thought to indicate cell viability. Later, however, it was postulated that fluorescing cell walls had not been formed during culture but were intact, undigested cell walls of cells presumed to be protoplasts at the time of culture initiation (see section 3.2.1). An interesting comparison can be made between the culture of mesocarp pieces on solid MSB media and the culture of single mesocarp cells in KP8 medium - while mesocarp pieces were incubated longer than seed sections before undergoing dedifferentiation and callus formation on solid media, individual cells isolated from the same tissue and incubated in a far more complex medium, were unable to dedifferentiate and proliferate.

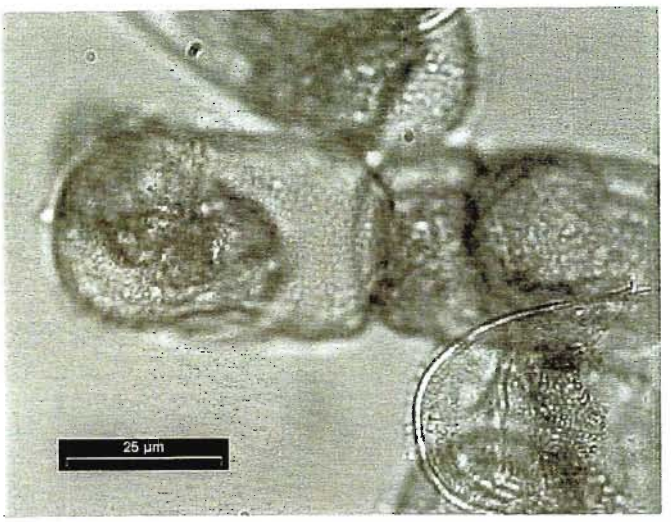
3.2.5 Isolation of protoplasts from cell suspensions

Because of their relatively homogenous cell population, cell suspensions are often used as source material for protoplasts. Blickle *et al.* (1986) reported on the isolation of protoplasts from callus tissue using a simple digestion medium (section 2.2.1) and, since callus tissue and cultured cells are not dissimilar, it was postulated that the application of the method of Blickle *et al.* (1986) would yield comparable results. Further, it was decided to test the efficacy of four

a



b



c

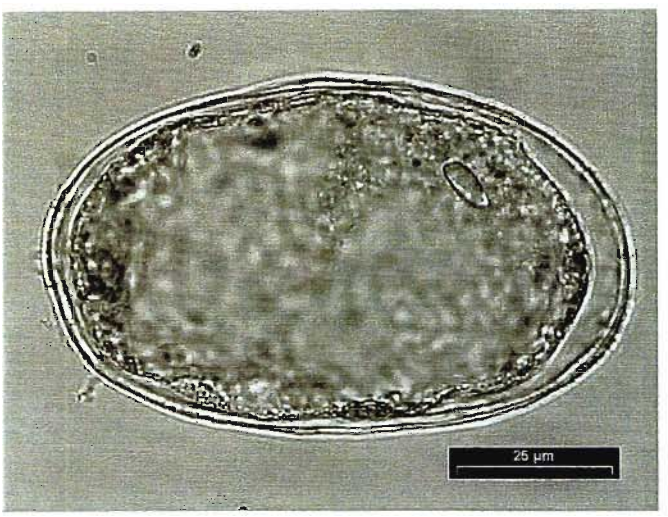
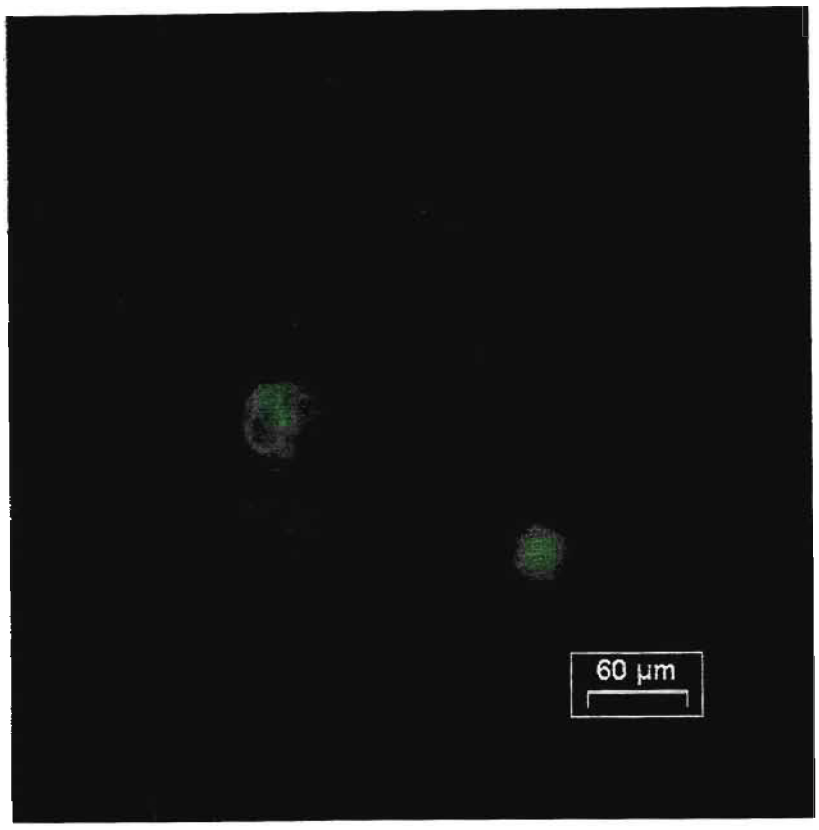
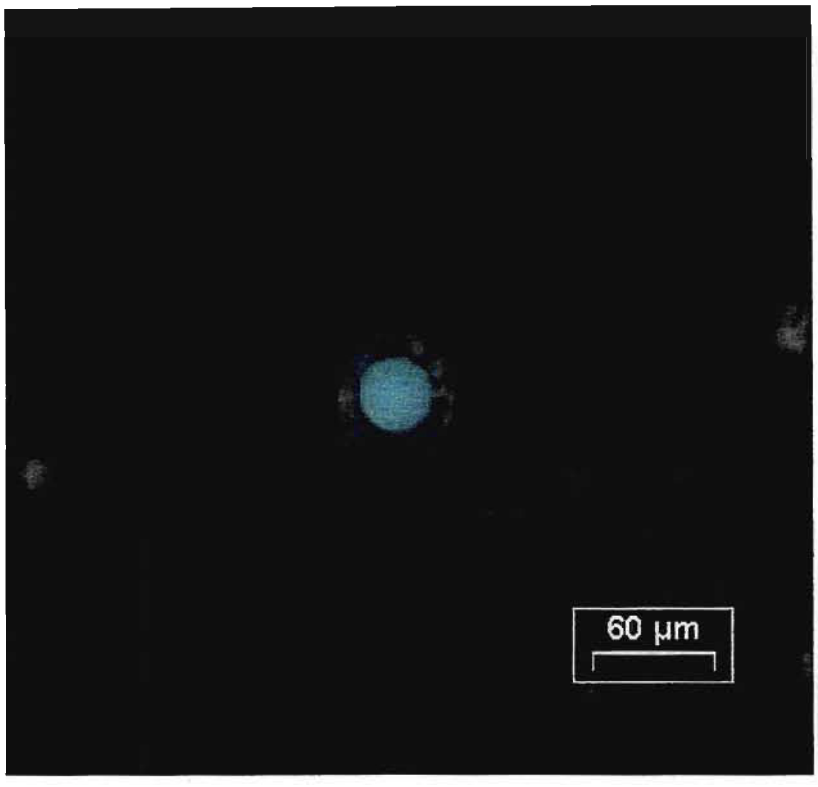


Figure 3.20

Micrographs illustrating typical cells and cell aggregates in suspension. a - c) Cells were of various shapes and sizes (a) and appeared densely cytoplasmic (b). Micrograph c shows a characteristic single cell with a dense cytoplasm and intact cell wall.



a



b

Figure 3.21

a and b) Fluorescing cells cultured in a microscope slide chamber in KP8 media containing 10 μgmL^{-1} FB28. No proliferation was observed during the 7 weeks of culture and it was postulated that any cell wall fluorescence recorded was probably from cells that had undigested cell walls at the start of culture.

other media used for the isolation of protoplasts from cell suspensions of other species (section 2.2.1).

No protoplasts were observed following incubation in any of the five media used. All treatments generally caused plasmolysis of the cultured cells (Fig. 3.22 a and b), a prerequisite for protoplast isolation, but cell walls almost always remained intact. Occasionally, spherical cells emitting low levels of fluorescence (Fig. 3.22 c) were observed but not in great numbers. Increasing the enzyme concentration in the digestion medium two-fold and four-fold, had no effect on protoplast yield.

3.3 SUMMARY

1. Isolation of protoplasts from avocado mesocarp seems to be more difficult than Percival *et al.* (1991) reported. Isolated cells were in various stages of cell wall degradation but very few cell walls were ever observed to have been completely digested.
2. Cell type ratios in purified suspensions approximated those found in intact fruit although a third cell type was regularly identified which was almost an intermediate between the multiple lipid bodied type 1 cell and the single lipid bodied idioblast.
3. Vital stains for testing cell viability have to be used carefully to avoid erroneous results. Exclusion dyes such as phenosafranin are probably best to determine proportions of viable cells although the fluorochrome stain FDA yields interesting and accurate results if a standardised time is used. FDA also offers the option of quantifying viability through measurement of fluorescence with a fluorimeter or extracting fluorescein and determining viability spectrophotometrically. AO is too subjective for use as a viability measure.
4. Mesocarp tissue is more recalcitrant towards callus induction than seed and embryo tissue probably due to its more differentiated physiological state.
5. Callus induced from any fruit explant excluding the pericarp is generally one of four types:

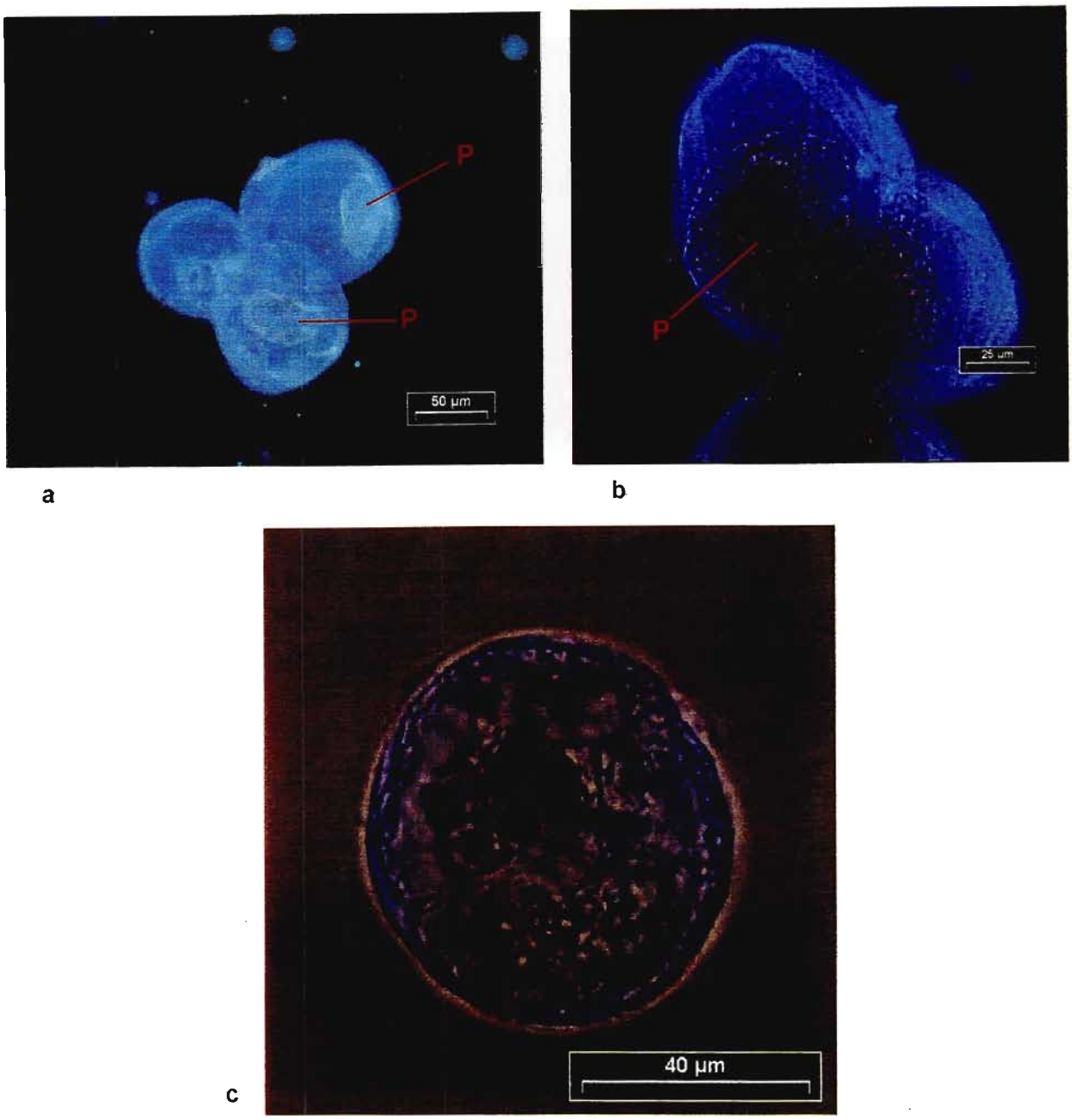


Figure 3.22

Micrographs of cultured cells after overnight incubation (20 h) in protoplast isolation media. The cells were stained with 10 gmL^{-1} FB28 dissolved in DMSO and viewed under UV (450-490 nm). a and b) Micrographs showing plasmolysed cells (P) with intact, fluorescing cell walls. c) Spherical cell emitting low levels of fluorescence indicating substantial cell wall removal. These cells were observed only occasionally and only in low numbers.

- Hard, white, crystalline callus which is not particularly conducive to subculture
- Wet, lumpy, friable callus which is the most amenable to subculture onto solid media as well as into liquid media for the initiation of cell cultures.
- Light, friable, dry callus which does not subculture onto solid media but forms an interesting structure on transfer to liquid media.
- Nodular callus which may or may not be the first step towards somatic embryo formation from undifferentiated cell masses.

6. Culture of excised embryos is extremely variable and probably reflects the complex genetic evolution of the top commercial cultivars.
7. Cell suspensions are relatively easy to initiate and maintain although generation times are surprisingly long compared to other species (cf Tobacco BY2 cell division cycle of 14 h (Hemmerlin and Bach, 1998)).
8. Culture of cells isolated from mesocarp tissue using various semi-solid and liquid methods incorporating KP8 protoplast culture media was unsuccessful.
9. Isolation of protoplasts from cell cultures using five different media, including one reported to yield positive results with avocado callus tissue, was also unsuccessful.

CHAPTER 4

INVESTIGATIONS INTO THE CONTRIBUTION OF THE ISOPRENOID PATHWAY TO GROWTH OF CELL SUSPENSION CULTURES

4.1 INTRODUCTION

The isoprenoid group of compounds in plants is composed of a wide variety of moieties with an equally diverse range of structures and functions. Chappell (1995) conveniently divided the isoprenoids into two categories – primary and secondary metabolites. The compounds categorized as primary metabolites are regarded as being essential for cell and plant viability and include sterols, carotenoids, growth regulators and the polyprenol substituents of dolichols, quinones and proteins (Chappell, 1995). Secondary metabolites, while not crucial for cell viability, mediate important interactions between the plant and its environment and include monoterpenes, sesquiterpenes and diterpenes. The isoprenoid biosynthetic pathway plays a fundamental role in synthesizing compounds that are of both structural (carotenoids and the side chain of chlorophylls for photosynthesis, the side chain of ubiquinone for respiration, sterols for membrane integrity and phytoalexins for defense) and regulatory (abscisic acid, brassinosteroids, gibberellic acid and the side chain of cytokinins) significance in plants (Cowan *et al.*, 1997).Together, these compounds are integral for proper morphogenetic expression during normal plant ontogeny (Fig. 4.1). The control of the isoprenoid pathway in plants has not been fully elucidated and many aspects remain controversial. It is generally agreed that the synthesis of mevalonic acid (MVA) from the reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is the first committed step in the pathway and therefore a major point of potential regulation (Bach, 1987; Gray, 1987; Moore and Oishi, 1994; Chappell *et al.*, 1995).

In avocado, however, it is the role of isoprenoid metabolites and their interactions with each other in the control of cell division and fruit ontogeny as well as the response of the pathway to exogenous (environmental) and endogenous

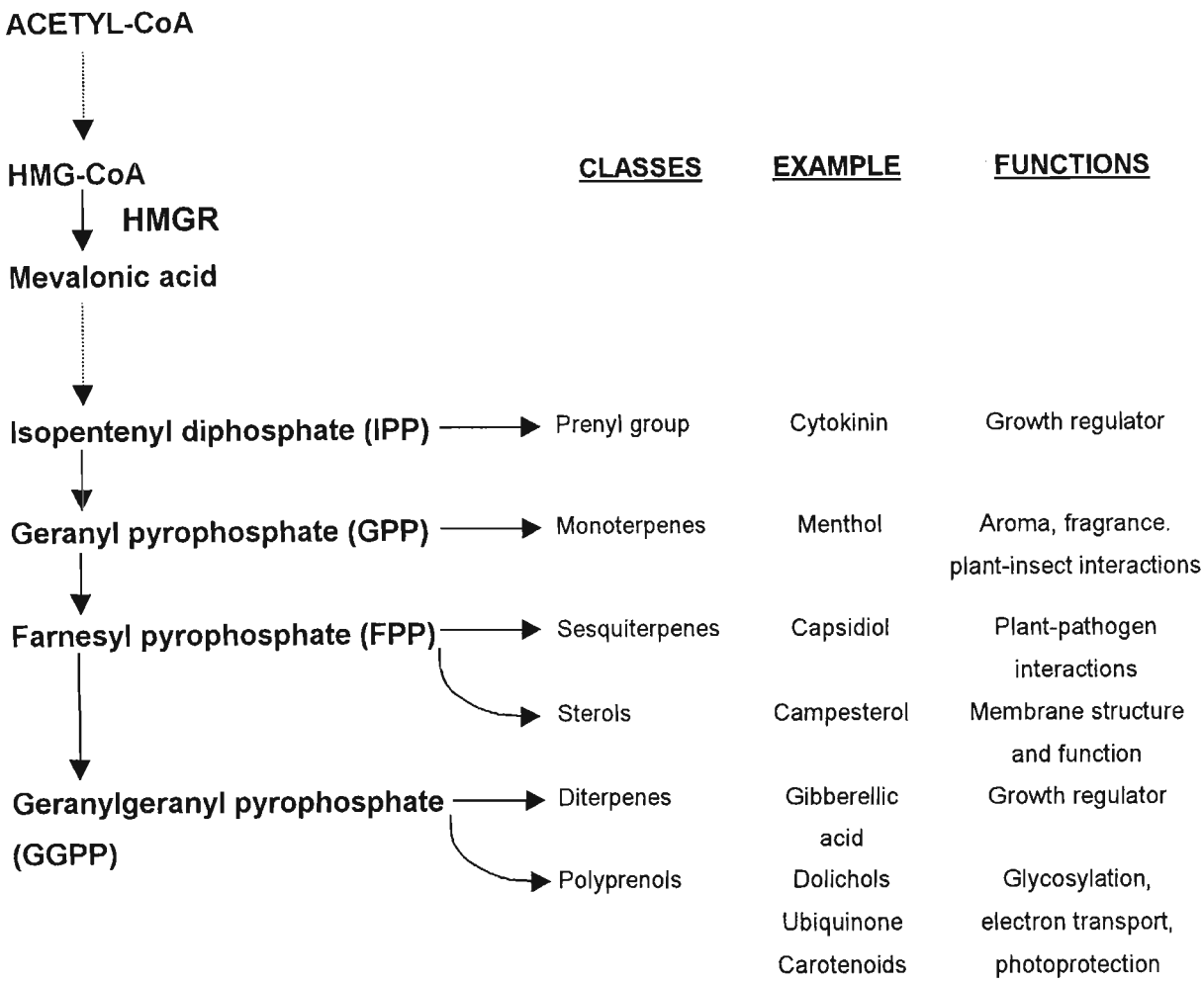


Figure 4.1

Schematic representation of the isoprenoid pathway showing intermediates and end products with their respective physiological functions. Broken arrows denote multiple reactions. The proposed rate limiting step of the pathway is the reduction of HMG-CoA to mevalonic acid by HMGR. (After Chappell, 1995)

(regulator levels) stimuli that is commanding attention. Recent work by Cowan *et al.* (1997) proposes that the appearance of the small fruit variant in 'Hass' avocado is a function of the interactions between sterols, CK, ABA and HMGR in fruit tissue. This conclusion was arrived at by analyzing small and normal phenotypes for elevated or depressed levels of ABA and HMGR and then treating phenotypically normal fruit during phases I, II and III of growth with various combinations of the HMGR inhibitor mevastatin and isoprenoid compounds. What they found was that, firstly, the growth of the small fruit variant was limited by cell number and that these fruit contained elevated levels of ABA and reduced levels

of HMGR compared to normal fruit, secondly, mevastatin treated fruit showed reduced growth and increased ABA content, thirdly, the effects of mevastatin could be partially reversed by co-injection with the sterol stigmasterol during growth phases II and III and, fourthly, co-injection with the CK iP reversed the mevastatin-induced retardation of fruit growth. Briefly summarized, their data suggest that an increased ABA level is central to the appearance of the small fruit and influences four areas of cellular function as illustrated in Fig 4.2. Obviously, each reaction does not occur in isolation and together they comprise a very complex, interactive system, the origin of which is still to be found.

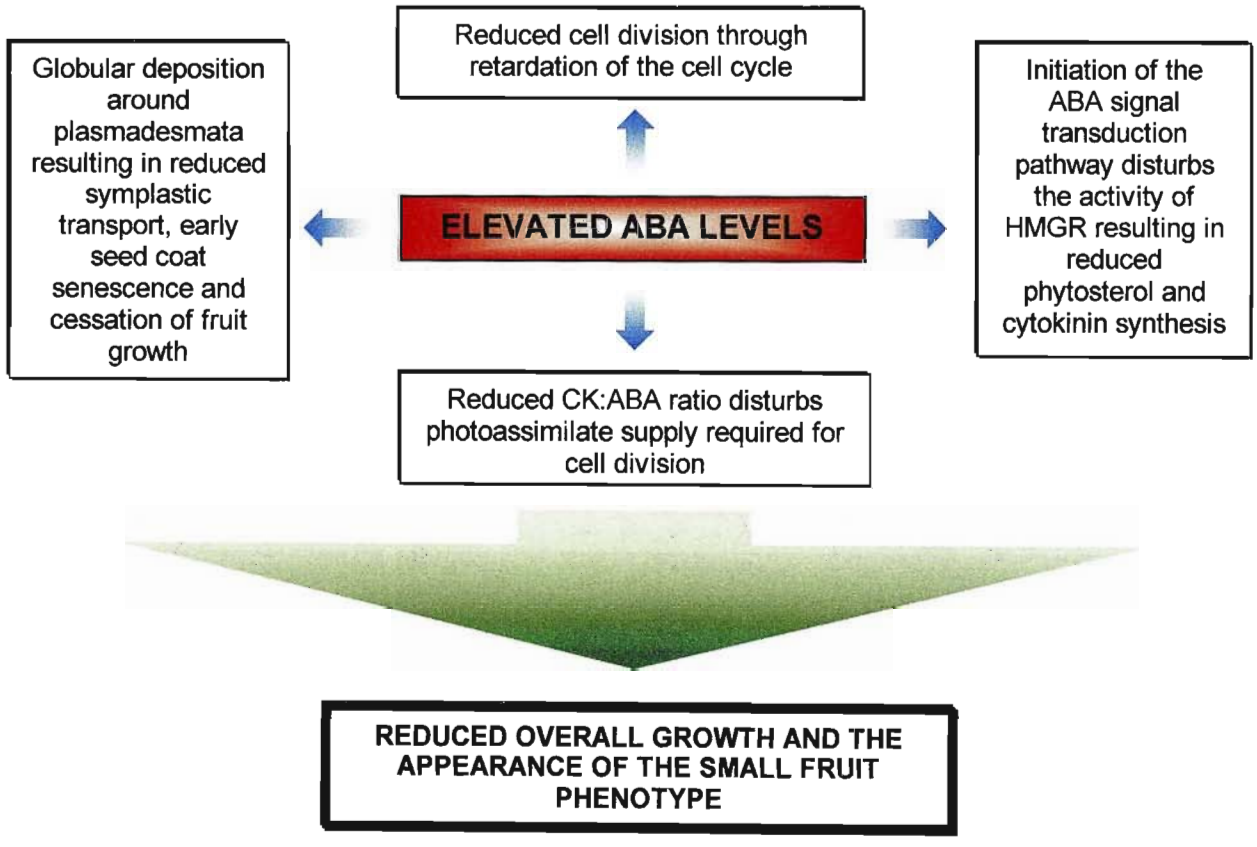


Figure 4.2

Schematic representation of a summary of the effects of increased ABA levels found in the small fruit variant of avocado reported by Cowan *et al.* (1997) and Moore-Gordan *et al.* (1998)

Nevertheless, while *in vivo* trials yield results from the source tissue concerned in the research, data may be confounded by biotic and abiotic stresses not

accounted for. The cell cultures developed in section 3.2.9 present an ideal system to test the responses of avocado fruit cells to mevastatin under controlled, defined conditions and thereby further our knowledge on the interactions of the components of the isoprenoid pathway and their influence on growth.

4.2 RESULTS

4.2.1 Investigations into population dynamics of 'Hass' avocado cell suspensions

Growth dynamics of cell cultures of 'Hass' avocado were studied in auxin and cytokinin dependent suspensions, derived from nucellar seed tissue of 8 – 9 month-old 'Hass' avocado fruit, treated with mevastatin. Various concentrations of mevastatin were used to test the response of the cultures and select a level to use in subsequent investigations. Both cell number, and settled cell volume (SCV) after 10 min, were used to monitor the growth response of the cultured cells. For cell counting, only intact cells that appeared viable were recorded and it was observed that some treatments displayed negative growth patterns that were not reflected in SCV measurements. It was therefore decided that all experiments would be monitored by cell number and not SCV.

Fig. 4.3 illustrates the growth response of cultured cells to various mevastatin concentrations. All levels of mevastatin elicited a response by day three. By day 10, cells treated with 0.01 μM mevastatin had recovered enough to show positive growth while by day 17, 0.1 μM and 1 μM mevastatin-treated cells began to grow positively. It is interesting to note that all treatments, besides the control, caused a severe decline in viable cell number in the first week after application. Thereafter, all cell cultures began to recover, albeit at different rates, and, only the 10 μM and 40 μM mevastatin-treated cells had failed to show growth above the inoculation density. At all stages during the experiment, cells without mevastatin produced a growth response that was significantly ($P<0.05$) greater than all other treatments. Cells treated with 0.01 μM mevastatin recovered fastest but did not always show a growth response that was significantly greater ($P<0.05$) than the other mevastatin concentrations (Fig. 4.3). If SCV was used to monitor

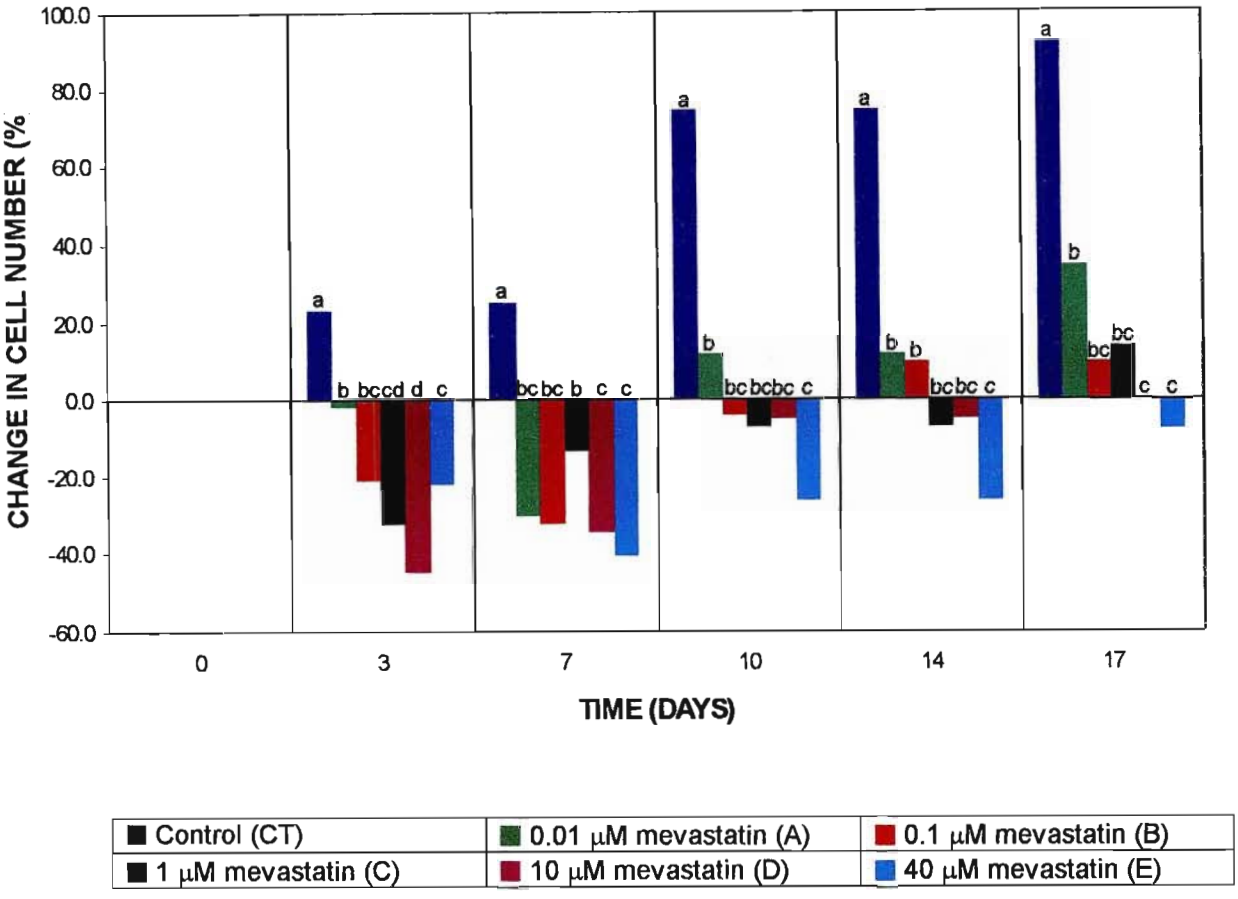


Figure 4.3

Growth responses of 'Hass' avocado cell suspensions to various mevastatin treatments. Bars with different letters within a particular measurement time are significantly different (P<0.05). Data are the mean of five replications and the experiment was duplicated. At all stages during the experimental period, the control growth values are significantly greater than all other treatments.

population growth, it might have been concluded that the 10 μM and 40 μM mevastatin treatments completely inhibited cell growth. However, Fig. 4.3 shows that, while growth is initially depressed (day 3 and 7), by day 10 the suspensions had shown signs of proliferation. Fig. 4.4 shows the results of FDA staining at day 17. The micrographs taken at a magnification of 2.5 x give a clear indication of the loss of viability of the cultures at the different mevastatin concentrations. Generally, the control, 0.01 μM and 0.1 μM treatments contained a large proportion of cells that were viable. The 1 μM mevastatin treatment caused a loss

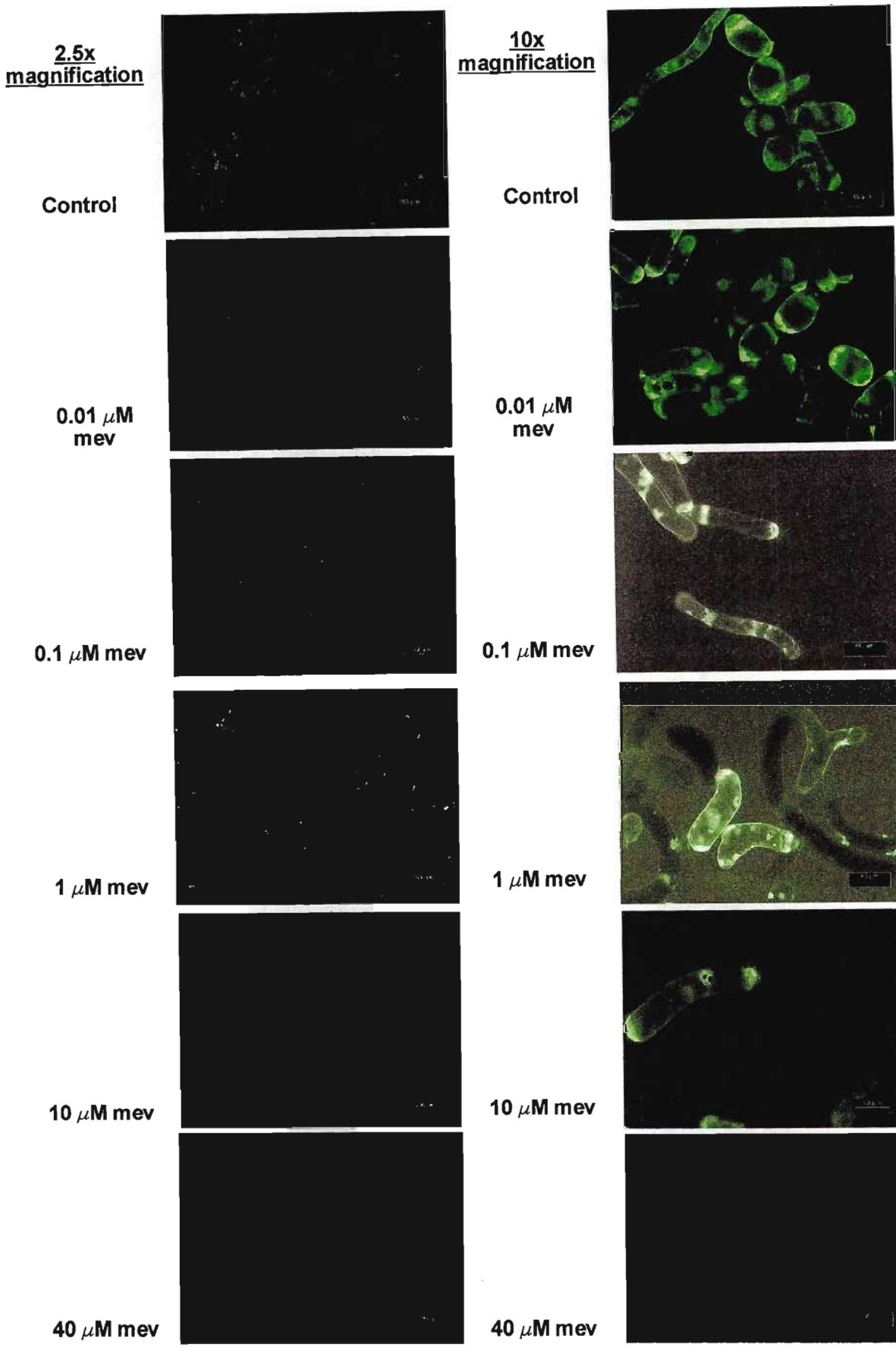


Figure 4.4

Micrographs of mevastatin-treated cells stained with 10 μgmL^{-1} FDA and viewed under UV (450-490 nm) on day 17 after mevastatin addition. General fluorescence decreases with increasing mevastatin concentration (2.5x magnification) indicating a general loss in cell viability. 10x magnification shows more specific treatment effects. Differences in background colour of the 10x magnification micrographs are due to alterations in gain (increase) and saturation (decrease) during image capture in order to show more clearly, fluorescing and non-fluorescing cells.

in cell integrity as shown in the 10 x magnification image (Fig. 4.4 h). The 2.5 x magnification micrograph of the 10 μM treatment indicated a substantial loss in viability compared to the previous four images although intact, viable cells are still present. The 40 μM mevastatin treatment resulted in an almost total loss of viability of the cultured cells, although several fluorescing cells that appeared intact were observed. Further to this result, it was observed that cells treated with 40 μM mevastatin turned brown overnight and appeared "oxidized" just as seed explants browned when exposed to commercial bleach. While graph values and FDA images corresponded on most data points, it was noted that the apparent increase in viable cells in treatments D and E (10 μM and 40 μM mevastatin) shown in the graph were not reflected in complementary micrographs to the extent one would expect since an increase in cell growth would be expected to be accompanied by a corresponding increase in fluorescing cells. This was not evident in the FDA images. Nevertheless, both sets of results indicate that inhibition of HMGR by sub-lethal levels of mevastatin (0.01 μM to 10 μM) causes growth retardation while higher concentrations (40 μM) appear to cause loss of culture viability and inhibition of growth. The question that can now be posed is, what metabolic product of MVA is most limiting to cell growth and, can cell growth be restored by the addition of isoprenoid compounds?

4.2.2 Reversal of mevastatin-induced inhibition of cell growth

In order to investigate the effect of isoprenoid compounds on growth responses of mevastatin-inhibited cells, two concentrations of mevastatin were chosen – a low concentration (1 μM) to minimize non-specific effects, and a high concentration (40 μM) for comparative purposes. To these two concentrations, DL-mevalonic acid lactone (MVL), farnesyl diphosphate (FDP) and stigmasterol were added. Cell counting and FDA staining were used to monitor population growth and cell viability respectively. The results are presented in Figs. 4.5 to 4.7.

Fig. 4.5 shows that the treatment of mevastatin-arrested cell suspensions of 'Hass' avocado with MVL and FDP, restores growth. Stigmasterol has little effect

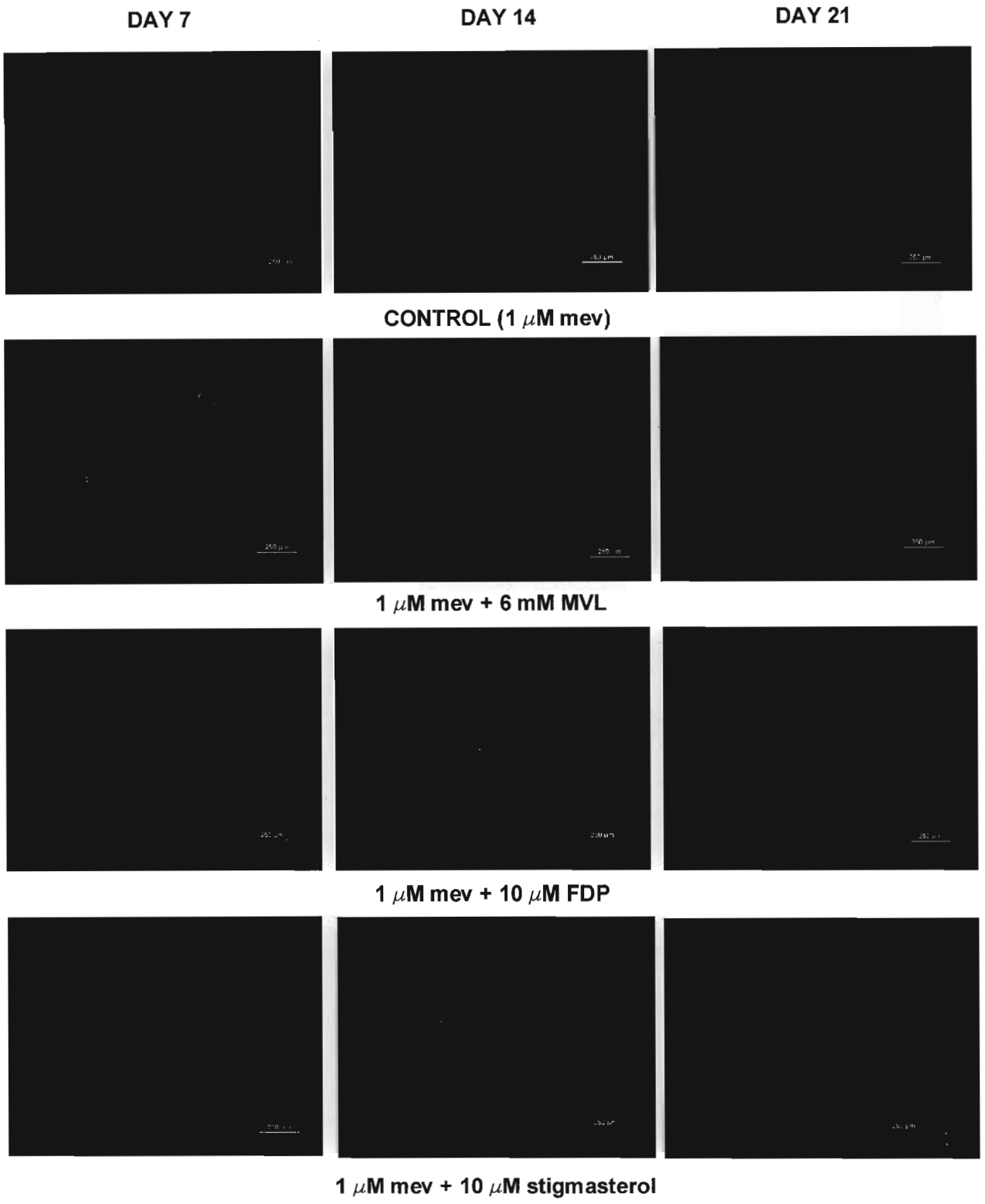


Figure 4.6

Migrographs at 7, 14 and 21 days after treatment with mevastatin (1 μM) and mevastatin + MVL (6 mM), FDP (10 μM) and stigmasterol (10 μM). Treatment effects, shown from the amount of fluorescence at each time period, conflicted with graphical trends depicted in Fig. 4.5 -control fluorescence remained fairly constant in agreement with graph trends but, fluorescence from the mev + MVL and the mev + FDP treatments decreased with time contrary to Fig. 4.5, while the mev + stigmasterol treatment appeared to display a larger proportion of fluorescing cells than any other treatment although growth responses in the chart showed little increase.

with stigmasterol, seemed to display a larger proportion of fluorescing cells than any other treatment although growth responses from Fig. 4.5 show little increase. The apparent anomalies may have arisen from unaccounted treatment effects and their interaction with FDA or possibly from the qualitative nature of monitoring which perhaps, should have been quantified by spectrophotometric analysis. The mode of action of FDA depends on the maintenance of membrane integrity. The FDA molecule is non-polar and permeates freely across the intact plasma membrane. Hydrolysis of FDA within the cell by non-specific esterases, yields fluorescein, which is a fluorescent, polar molecule which cannot cross the lipophilic plasma membrane. Fluorescein thus accumulates in the intact cell and emits fluorescence when excited by light in the UV range (Rotman and Papermaster, 1966; Larkin, 1976). Perturbations in membrane integrity or the presence of conditions which damage the cell without disrupting its morphological integrity (such as aging or treatment with surface agents) result in reduced, or lack of fluorescence (Rotman and Papermaster, 1966). As mentioned before, the isoprenoid pathway is integral to cell survival, and any disruption may cause a cascade of events affecting many areas of cell functioning. A co-treatment of cells with mevastatin and an isoprenoid compound, may result in conditions in which FDA is not metabolised and/or fluorescein does not fluoresce or remain in the cell. This might explain the lack of fluorescence observed from cells co-treated with mevastatin and MVL and FDP.

Fig. 4.7 shows the response of cells treated with 40 μM mevastatin plus MVL, FDP and stigmasterol. Again, MVL and FDP restore growth to the cultures while stigmasterol seems to induce a partial recovery although graph values are not significantly different from the control except on day 14. FDA images (not shown) did not show any significant fluorescence in any of the treatments from day 7 although graph trends indicate a growth response. It was noted, on leaving FDA treated samples overnight, that a diffuse fluorescence appeared in the incubation media. While it is accepted that a diffuse fluorescence always appears in cells and media after staining with FDA, it would be interesting to see if cellular response under the defined treatments was not temporally affected and/or if enzymatic action disrupted.

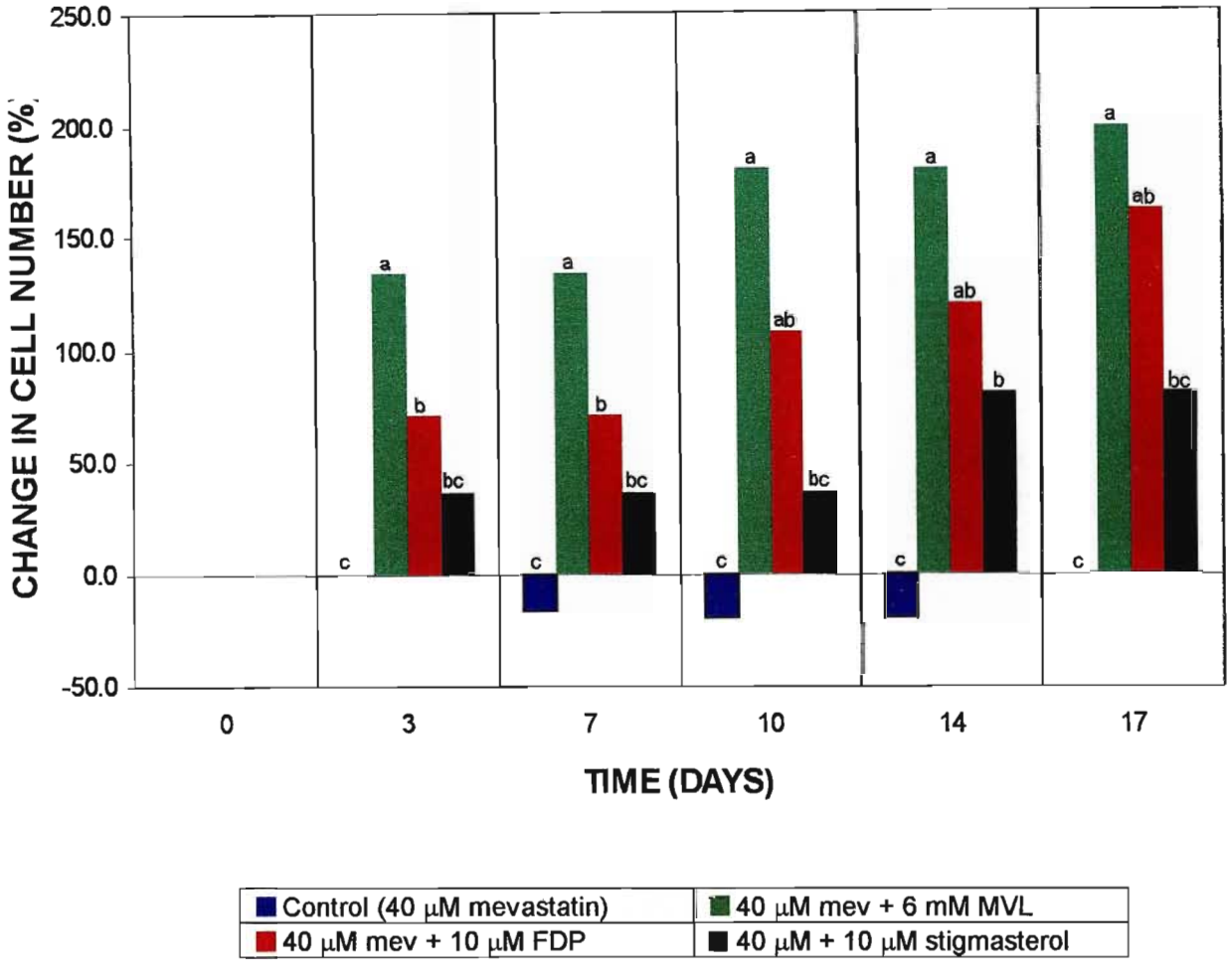


Figure 4.7

Growth responses of 'Hass' avocado cell suspensions to co-treatment with 40 μM mevastatin and 6 mM MVL (■), 10 μM FDP (■) and 10 μM stigmasterol (■). Bars with different letters within a particular measurement time are significantly different (P<0.05). Data are the mean of three replications and the experiment was duplicated. Growth responses of cell populations treated with MVAL and FPP are significantly greater than the control and stigmasterol treatment. Abbreviations, mev = mevastatin, MVL = DL-mevalonic acid lactone, FDP = farnesyl pyrophosphate.

4.3 SUMMARY

1. Mevastatin inhibits cell growth in 'Hass' avocado cell suspensions.
2. Treatment of mevastatin-inhibited cells with MVL and FDP restores growth.

3. Treatment of mevastatin-inhibited cell cultures with stigmasterol partially restores growth although growth responses are not always significantly different from the control.
4. FDA is a useful stain for monitoring cell viability but treatment effects may produce erroneous results.

CHAPTER 5

GENERAL DISCUSSIONS AND CONCLUSIONS

5.1 GENERAL DISCUSSION

The results of this study showed that protoplast isolation from the mesocarp of avocado was difficult and not reproducible. Callus induction from seed tissue, however, was relatively easy and provided good source material for the initiation of cell suspension cultures. It was also shown that these cell suspensions could be maintained through several passages of sub-culture and that the cells were metabolically active. Subsequent manipulation of cellular mechanisms supported recent evidence suggesting a key role for isoprenoid derivatives in the control of cell growth and development in avocado (Cowan *et al.*, 1997; Moore-Gordon *et al.*, 1998).

5.1.2 Development of an *in vitro* culture system from avocado fruit

Plant protoplasts have been described as cells that have had their cell walls removed, usually by mechanical or enzymatic action. The contents of the cell are separated from the immediate external environment by the phospholipid matrix of the plasma membrane only. This state places protoplasts under extreme osmotic stress and to survive, they have to be maintained in an isotonic nutrient medium. The structure of the protoplast, with the plasma membrane fully exposed, presents plant biologists with a unique system for studying physiological, genetic and biochemical phenomena. Since the first report on the regeneration of a complete plant from isolated protoplasts (Takebe *et al.*, 1971), research concerning these cells has focussed on their genetic manipulation and subsequent regeneration of transgenic plants. This is indeed a valuable scientific contribution but, over 30 years, has been met with limited success with regard to producing useful transformants. One wonders if, perhaps, more research was directed towards the elucidation of physiological and genetic properties associated with the cellular state of the protoplast, more efficient protocols, with more accurate predictions of the

outcome, would be developed. With several authors reporting that protoplasts respond to environmental and hormonal stimuli in a similar manner to that of the tissue from which they were derived (Hooley, 1982; Percival *et al.*, 1991), it is surprising that more work has not been carried out using this system. A particular case in point is that of fruit tissue. The fruit developmental program, involving the molecular, cellular and physiological control mechanisms and their coordination, remains an enigma despite centuries of intensive genetic selection (Gillaspy *et al.*, 1993). In avocado, Percival *et al.* (1991) showed that protoplasts isolated from mesocarp tissue, had potential for use as a model system for studying the ripening process. They reported that protoplasts were readily obtained from mature fruit before, during and after the climacteric. The present investigation showed that, while the plasmalemma was readily degraded to release single cells, the cell walls of individual cells were not readily hydrolysed to yield protoplasts. Percival *et al.* (1991) noted that residual cell wall material was still visible on some cells after incubation even though most of the cells were spherical. The data obtained by staining with FB28 in this study revealed that even "spherical cells" could still have intact cell walls. No doubt Percival *et al.* (1991) did isolate protoplasts but differences in isolation media and purification techniques between the two studies make comparisons difficult. Nevertheless, one can pose the question of the present study, why were no protoplasts isolated?

In the isolation of protoplasts, there are several factors which contribute to the success or failure of the procedure. The source tissue is of prime importance – its structure, age and physiological state determine to a large extent, the effect of the digestion solution. The mesocarp of avocado fruit consists primarily of two cell types according to Platt-Aloia *et al.* (1980). 2% of the tissue volume is composed of idioblast oil cells while the remainder comprises simple parenchyma cells. The specialized oil cells are surrounded by a complex cell wall consisting of three layers – the outer primary cellulosic cell wall, a secondary suberin layer and an inner tertiary cellulosic cell wall (Platt-Aloia *et al.*, 1983). The parenchyma cells have a simple cell wall structure enclosing the contents consisting of only a primary cellulosic wall. During ripening, an ordered series of events takes place which results in the

softening of the fruit. These events are carried out, in part, by hydrolytic enzymes not dissimilar to those used in the preparation of protoplasts. It would be useful to study these processes to gain a better understanding of the structure of mesocarp tissue and its degradation during ripening for comparative purposes. Structurally, the avocado mesocarp tissue begins to soften by disruption of the cell wall matrix followed closely by dissolution of the middle lamella (Platt-Aloia *et al.*, 1980). As ripening proceeds, the middle lamella is totally broken down and structural unity of the cell walls is lost. Cellulose microfibrils shorten and become loosely packed. Complete degradation of the cell wall was only occasionally observed by Platt-Aloia *et al.* (1980) in post-climacteric, overripe fruit. In these cases, however, suspended organelles could be seen through the plasma membrane of what was essentially an *in vivo* protoplast. Studies into changes in hydrolytic enzymes over the same time period by Awad and Young (1979), revealed that cellulase activity increased prior to an increase in polygalacturonase activity. Hatfield and Nevins (1986) showed that activity of avocado cellulase is limited to hydrolysing specific β-D-glucans consisting of four or more glucosyl residues. They reasoned that cellulase hydrolysed cellulose microfibrils in the cell wall resulting in general instability and alterations in hydrogen bonding between other polysaccharides. This action exposes polygalacturons embedded in the wall matrix to specific pectinases which are expressed shortly after cellulase (Awad and Young, 1979). Based on these results, Bower and Cutting (1988) surmised by stating that early stages of softening appear to be due to cellulase while the latter stages were due to polygalacturonase. Since the cell wall seems to be degraded to a considerable extent during the natural course of ripening, it is reasonable to suppose that no structural anomaly prevented isolation of protoplasts in the present study.

While no specific age can be attached to source tissue for optimum isolation results, it is generally thought that young tissue with actively dividing cells is best (Eriksson, 1985; Bengochea and Dodds, 1986). The tissue used in the present investigations was excised from 7 – 8 month-old, pre-climacteric fruit

harvested from healthy trees. At this stage in the avocado developmental program, fruit is nearing the end of exponential growth. Cell division is occurring but at a reduced rate relative to earlier stages. As a result, mesocarp cells are in various stages of maturity ranging form immature (newly divided) to mature (differentiated). This provides a selection of cells useful for investigations into many aspects of fruit development and ripening with seemingly no effects prohibiting cell wall degradation.

As with the property of source tissue age, optimum physiological status of cells cannot be generally defined. The intended use of protoplasts and the response to be measured, will specify what physiological state is required. In fruits, as with all other plant tissue, age and physiological status are intrinsically related. Fig. 3.1 suggests that the stage of development at which fruit were harvested for protoplast isolation, physiological events associated with auxins (AUX), gibberellins (GA) and cytokinins (CK) were being down-regulated while ABA-mediated reactions, linked to the induction of ripening processes, were being initiated. At this point in avocado ontogeny, mesocarp tissue is in a state of transition where developmental programs are drawing to a close and ripening and senescence programs are being initiated. The cell is still physiologically active but confounding effects with regard to *in vitro* manipulations, such as high hormone concentrations, are minimal. Negative interactions between ABA and hydrolytic enzymes in the digestion medium are unlikely since during normal ripening, high levels of ABA occur concurrent with the expression of cellulase and polygalacturonase (Bower and Cutting, 1988). Thus, it would seem that cell structure, tissue age and physiological state had no negative effect on protoplast isolation in the present investigation. It is more likely that a component or an interaction between components in the digestion medium is / were responsible for the lack of success in protoplast isolation.

Comparison of the composition of the isolation medium used by Percival *et al.* (1991) with that used in this study, reveals that the only apparent differences exist in the type and source of hydrolytic enzymes. Fitzsimmons and Weyers (1985) state that enzyme mixtures used for the isolation of protoplasts are

products of undefined composition. Commercial enzyme products are widely considered as products of some uniformity although manufacturer's specifications rarely indicate details of the purification procedures used nor the total chemical composition of the preparation. In a trial comparing the activity of seven different batches of a commercial cellulase, cellulysin, and its modulation by various additives, Fitzsimmons and Weyers (1985) showed that a considerable degree of variation in enzyme efficacy existed. Using guard cell protoplast (GCP) release from *Commelina communis* epidermal tissue as a response variable, they categorized the seven batches into three classes according to their activity (cellulase units (U) mg^{-1}) and efficiency (yield of GCP at a particular concentration). In cases where cellulase activity was high (ca. 3 Umg^{-1}), low concentrations of cellulysin gave high yields of GCP within 4 – 6 h. These were designated "class 1" batches. The remaining batches tested had slightly lower activities (1.2 – 2.0 Umg^{-1}) and were found to be either inefficient (GCP release only at high concentrations – "class 2" batches) or inactive ("class 3" batches). Furthermore, Fitzsimmons and Weyers (1985) found that the inactivity of class 3 batches could be partially reversed by the addition of a pectinase preparation, pectolyase Y-23. From this, they suggested that the absence or inactivation of pectinase enzymes normally present in class 1 batches of cellulysin was the cause of inactivity in class 3 batches. They also noted that other pectinases, including Sigma pectinase, did not enhance GCP release in conjunction with class 3 cellulysin. These results are particularly significant to the present study. The cellulase used in the isolation of avocado mesocarp protoplasts was obtained from Roche Biochemicals (formerly Boehringer), Germany and had a labelled activity of 0.5 Umg^{-1}. While Fitzsimmons and Weyers (1985) mentioned this cellulase, no comparisons with the cellulysin batches were reported. However, because of the comparatively low labelled activity and the negative results obtained even at high concentrations (4% w/v), it is reasonable to suggest that this preparation could fit into the class 3 category. The pectinase used was obtained from Sigma and, although Fitzsimmons and Weyers (1985) reported that this preparation did not improve GCP yield in their investigation, the fact that individual cells were obtained from mesocarp sections suggests that the middle lamella had been degraded. The middle lamella in avocado mesocarp

tissue has been shown to contain considerable amounts of pectin (Platt-Aloia *et al.*, 1980) and therefore it can be postulated that Sigma pectinase was active in the digestion medium. Interactions between isolation media components and enzyme preparations are unclear. It has been reported that 1,4-dithiothreitol (DTT) improves GCP isolation with increasing concentration but that protoplast viability decreased at high concentrations (Fitzsimmons and Weyers, 1985). Bovine serum albumin (BSA) is added to many digestion solutions as an alternative substrate for proteases, contained in cellulase preparations, which may disrupt plasma membrane integrity and subsequent viability of protoplasts. It is unlikely that in this study there were significant interactions between other components of the isolation media since inorganic salts and osmoticum were identical to those used by Percival *et al.* (1991). Thus, it can be concluded that the apparent inability to isolate protoplasts from mesocarp of avocado fruits was due to the cellulase enzyme preparation which appeared to be inactive.

Failure to generate a viable protoplast system for use in metabolic studies prompted the author to investigate cell cultures as an alternative. The initiation of cell cultures requires an intermediate step of callus induction from desired explants.

Callus is described as relatively undifferentiated tissue consisting primarily of parenchymatous cells (Caponetti, 1996). It usually develops from damaged plant tissue as a result of the induction of an as yet ill-defined wound response followed by a growth response (Allan, 1991). The wound response is characterised by an increase in metabolic activity but limited cell division while the growth response is recognized by sustained cell division dependent on an exogenous supply of auxin (Allan, 1991). Callus tissue is morphologically more uniform than a complete plant and provides a potentially useful system for studying basic research problems in cell biology. In addition it provides material from which cell suspensions can be obtained as well as being a starting point for plant regeneration.

In avocado, callus induction and culture has been established for many explants (Mohammed-Yasseen, 1992). Of particular interest to researchers, was the ability of mesocarp tissue to produce callus. It was reasoned that this phenomenon was due to sustained cell division activity in mesocarp tissue (Schroeder, 1958). To some extent this hypothesis has been corroborated but Blumenfeld and Gazit (1971) showed that mesocarp tissue was dependent on exogenous CK for cell division. In addition, seed explants at the same stage of fruit development did not require exogenous CK for callus growth and endosperm extracts showed high CK activity. Blumenfeld and Gazit (1971) concluded that CK were produced in the seed and postulated that mesocarp cells were supplied with CK from either the tree or the seed via the seed coat. The aim of the callus culture stage in the present study was to obtain material to initiate cell cultures. Nevertheless, results from 7 – 8 month-old fruit (see sections 3.2.2.1 and 3.2.2.2), confirm those of Blumenfeld and Gazit (1971) to a certain extent and lend support, albeit obtuse, to recent hypotheses for the involvement of CK in appearance of small 'Hass' fruit (Cowan *et al.*, 1997; Moore-Gordon *et al.*, 1998) through the elevation of ABA levels. The apparent elevated response of seed tissue compared to mesocarp tissue, suggests either a high endogenous level of hormone already present, or an increased sensitivity to applied growth regulators. Studies by Blumenfeld and Gazit (1971) showed that fifth generation callus derived from seed tissue had CK-like activity needed for cell division but that callus derived from mesocarp tissue had an absolute requirement for CK. The small fruit phenotype of 'Hass' avocado is characterised by a senescent seed coat, decreased symplastic and apoplastic solute movement and a reduced CK:ABA ratio (Cowan *et al.*, 1997). In light of this, it is tempting to suggest that if CK derived from seed tissue are used in mesocarp cell division, then physical barriers to their transport, such as a non-functional seed coat or gated plasmodesmata (Moore-Gordon *et al.*, 1998), might contribute to the appearance of the 'Hass' small fruit phenotype. It was considered that manipulations of callus derived from the respective tissues might yield further information. The proposed hypothesis is, however, oversimplified and it is recognized that far more complex interactions do occur. Even so, callus tissue remains a useful material for basic research. One problem with the use of callus culture as a

research system is that it is heterogenic and has a relatively slow response time. These two variables can be improved, although not eliminated, by the transfer of callus to liquid media and the formation of cell cultures.

In this study, one type of callus, identified by its morphological characteristics, was used for initiating cell cultures. The callus was "wet and lumpy" (Fig. 3.12a) and separated easily in solution. Cell suspensions were started from callus derived from seed tissue since this showed fastest growth and higher amenability to liquid culture. Growth of most cell cultures, as measured by increases in fresh or dry weight, or cell number, usually follows the generalized form of a sigmoidal curve (Lindsey and Yeoman, 1985). Avocado cell suspensions were no exception – the cultures showed a lag phase of ca. 10 d and an exponential phase of ca. 12 – 15 d followed by a stationary phase. In comparison to other plant species (*Daucas carota*, *Glycine max* and *Nicotiana tabacum*), this growth cycle is long (Gould, 1984) and is probably a function of a sub-optimal nutrient composition coupled with a changing physical environment that is not corrected for (e.g. a decline in pH). Interestingly, cell cultures were observed to grow in the absence of CK (0.2 mgL^{-1} 2.4D only) lending support to Blumenfeld and Gazit's (1971) suggestion that seed tissue produced CK *in situ* although this was not measured. However, further trials showed this property to be variable – a function, perhaps, of genetic heterogeneity among explant tissue. It was therefore decided to use a medium containing both AUX and CK and this proved to be maintainable through subculture and reproducible from newly formed callus. As such, these cultures presented a potential system for use in metabolic research studies and the following section discusses cellular response of avocado cell suspensions to various chemical stimuli.

5.1.2 Investigations into the contribution of the isoprenoid pathway to growth of cell suspension cultures

Cell division is probably one of the most fundamental properties of biological systems. Growth and development of all plant tissues is a primary function of cell replication (Hemerly *et al.*, 1999). The events governing the control of cell

cycle progression are, therefore, of immense interest. The plant cell cycle is described in terms of four phases, G_1, S, G_2 and M (Fig. 5.1). Each phase is characterised by a particular set of biochemical and cellular activities. Briefly, nuclear DNA is prepared for replication in G_1; during S, DNA is replicated and during G_2 the cell prepares for mitosis (Taiz and Zeiger, 1998). The mechanism regulating the progression of cells through the cell cycle is highly conserved. The key components are a family of enzymes known as cyclin-dependent protein kinases (CDK). CDK activity is purported to be regulated through at least two mechanisms. First, the interaction with regulatory subunits known as cyclins which are classified as either mitotic or G1 depending on the period in the cell cycle during which they are expressed (Fig. 5.1). CDKs are inactive in the absence of an associated cyclin. Cyclin synthesis and destruction, therefore, controls CDK activity. The second mechanism of CDK regulation involves post-transitional modifications by reversible phosphorylations of key amino acid residues in CDK proteins (Taiz and Zeiger, 1998; Hemerly *et al.*, 1999). The interactions between CDKs, cyclins and phosphatases regulate the progression of cells through the cell cycle (Fig. 5.1). What is of interest to the present study is the involvement of isoprenoid derivatives in the initiation and maintenance of the cell cycle. Products of the isoprenoid pathway, such as CK, ABA and sterols are purported to be involved in regulatory mechanisms associated with cell division (Gillaspy, 1993; Taiz and Zeiger, 1998). While evidence for a direct cell division response to these compounds is obscure, Jacobs (1995) notes that the CDK-cyclin driven cell cycle system is linked to the down-stream ends of signaling cascades, the up-stream components of which interact directly with growth factors. By specifically inhibiting the key enzyme in the isoprenoid pathway, HMGR, it was hoped that the interactions between isoprenoid compounds and cell division in avocados could be investigated.

It was shown that cell division was arrested in avocado cell suspensions by the addition of mevastatin. Similar results have been obtained in tobacco cell cultures (Crowell and Salaz, 1992), tomato fruit (Narita and Gruissem, 1989) and whole avocado fruit (Cowan *et al.*, 1997). Crowell and Salaz (1992) used

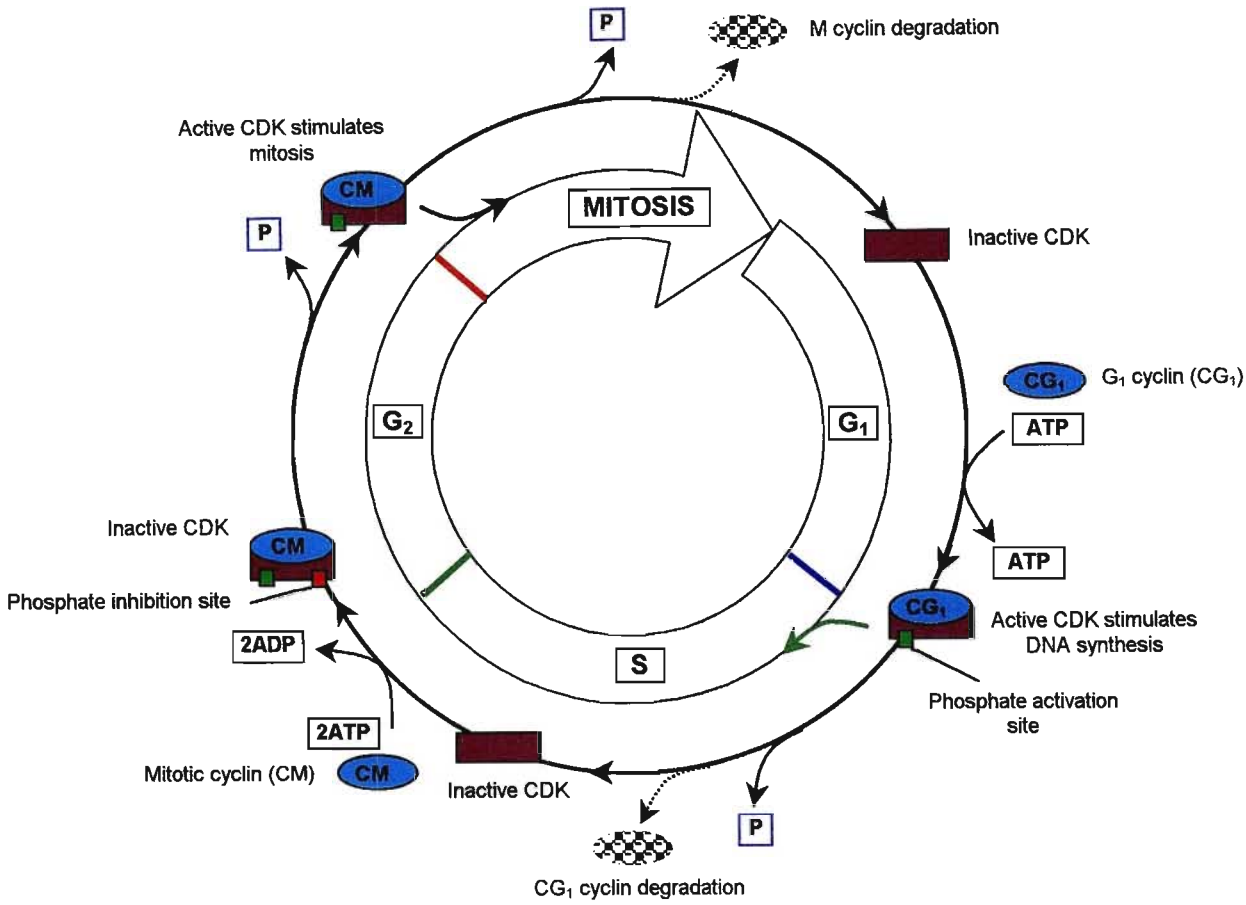


Figure 5.1

Cell cycle regulation by cyclin-dependent protein kinase (CDK). During G_1, CDK is in an inactive form. CDK becomes activated by binding to G_1 cyclin and undergoing phosphorylation at the phosphate activation site (■). The activated CDK-cyclin complex allows the transition to the S phase. At the end of the S phase the G_1 cyclin is degraded and the CDK is dephosphorylated resulting in an inactive form of CDK. The cell enters G_2. During G_2 the CDK binds to the mitotic cyclin (CM) and at the same time is phosphorylated at both the phosphate activation site and the inhibition site (■). The CDK complex is still inactive because the inhibitory site is phosphorylated. The CDK complex is activated when the phosphate is removed from the inhibitory site by a protein phosphatase. The activated CDK then stimulates the transition from G_2 to mitosis. At the end of mitosis the CM is degraded and the remaining phosphate is removed and the cell enters G_1 again. (After Taiz and Zeiger, 1998)

CK autonomous tobacco cultures and showed that the effects of lovastatin (an analogue of mevastatin) could be reversed by the addition of the CK, kinetin. Similarly, Cowan *et al.* (1997) reported that the inhibitory effects of mevastatin on avocado fruit growth could be completely reversed by co-treatment with iP.

The biosynthesis of endogenous CK is purported to involve the transfer of an isopentenyl group from dimethylallylpryophosphate (DMAPP) to adenine monophosphate (AMP) at the N^6 position (Taiz and Zeiger, 1998). Thus the observations of Crowell and Salaz (1992) and Cowan *et al.* (1997), might suggest that inhibition of HMGR results in a decrease of available IPP and an accompanying reduction in CK biosynthesis. The involvement of CK in cell cycle activity is complex but it has recently been observed in *Arabidopsis*, that CK stimulate the production of a gene encoding a G_1-type cyclin named δ3-cyclin (Soni *et al.*, 1995). This cyclin is thought to interact with the major cell cycle CDK which is encoded by the gene cdc2 which, in turn, is regulated by auxin (Jacobs, 1995). The cdc2CDK-δ3-cyclin moiety drives the cell through G_1 (Taiz and Zeiger, 1998). Furthermore, Zhang *et al.* (1996) postulated that CK controls the activity of a phosphatase involved in regulating the cdc2CDK in G_2 through the removal of an inhibitory phosphate group. Thus, it could be proposed that the reduction in endogenous biosynthesis of CK by the inhibition of HMGR is a major cause of reduced cell division.

Mevastatin-induced arrest of cell division in avocado cell suspensions was completely reversed by co-treatment with the isoprenoid compounds MVL and FDP. MVL is the immediate product of the reduction of HMG-CoA by HMGR and undergoes phosphorylation and decarboxylation to generate IPP, the monomer building block of all isoprenoid compounds (Chappell, 1995). It is not surprising therefore, that the addition of MVL reversed the effects of mevastatin. Crowell and Salaz (1992) observed the same effect in tobacco cell cultures. On the other hand, the cell growth response to FDP poses several questions. FDP is an isoprenoid compound appearing after GPP in the isoprenoid pathway. It forms a branch in the pathway leading to sesquiterpene and sterol derivatives (Chappell, 1995). Thus, the addition of FDP only would not satisfy substrate demand for compounds synthesized higher in the pathway. Why then, is there complete reversal of mevastatin-induced cell cycle arrest? Three possibilities are apparent. First, it is possible that multiple HMGCoA reductases exist in plant cells and that their sensitivity to mevastatin differs. This suggestion was put forward by Crowell and Salaz

(1992) in an attempt to explain the specificity of low concentrations of lovastatin. They proposed that the different HMGRs were dedicated to different aspects of isoprenoid metabolism and were separated by intracellular compartmentalization. The fact that subcellular sites of synthesis of isoprenoid products differ (Kleinig, 1989), lends credence to this theory. In addition, mevastatin is thought to have only cytosolic inhibitive activity (Bach and Lichtenthaler, 1983; Bach, 1987) and therefore plastid-localized HMGR may still be active. Further evidence in support of this theory comes from the characterisation of several genes encoding different isoforms of HMGR (Choi *et al.*, 1992). Differential expression of these genes results in metabolic switching of the isoprenoid pathway at the level of FDP. These results also provide evidence supporting the existence of discrete channels which direct the flow of intermediates to products further down the pathway which are preferentially needed for specific tasks (Chappell *et al.*, 1995).

Secondly, FDP and its subsequent isoprenoid derivative, geranylgeranyl diphosphate (GGPP) are involved in protein prenylation. Protein prenylation is a post-translational protein modification, the products of which play key roles in signal transduction, cytoskeletal and nuclear architecture, membrane transport and the progression through the cell cycle (Rodriguez-Concepcion *et al.*, 1999). Prenyltransferases catalyze the reaction in which FPP or GGPP are covalently bonded to cysteines near the C-termini of targeted proteins (Rodriguez-Concepcion *et al.*, 1999). Three different enzymes are associated with prenylation in animals and yeasts – a single farnesyl transferase (Ftase) and two geranylgeranyl transferases (GGTase-I and II). In plants, the presence of Ftase and GGTase-I and GGTase-II (Rab-GGTase) has recently been confirmed (Randall *et al.*, 1993; Schmitt *et al.*, 1996; Yalovsky *et al.*, 1996). While there is little information on plant GGTases, Ftase activity in plants has been well characterized. In tomato, Ftase activity was found to be highest in stems, roots and young fruit with lower activities present in mature fruit, leaves, seedlings and apical buds (Schmitt *et al.*, 1996). In pea, transcript levels of GGTase-I and Ftase were found to be highest in root nodules in which cells are actively dividing (Qian *et al.*, 1996). Furthermore, addition of an Ftase inhibitor, manumycin, to tobacco BY-2 cell cultures

blocked cell cycle progression giving rise to speculation that Ftase plays a role in cell division (Morehead *et al.*, 1996). The process of prenylation is not considered to be regulatory because the covalent attachment of FDP or GGPP is non-reversible (Rodriguez-Concepcion *et al.*, 1999). However, the availability of these substrates may limit protein modification. Thus, perturbations in isoprenoid synthesis through mevastatin-induced HMGR inhibition, would affect protein prenylation at the cell level. Ftase protein substrates involved in the mediation of regulatory events in cell division have not been identified although it has been reported that Ftase inhibition blocks cell cycle progression (Morehead *et al.*, 1996). It would be reasonable, therefore, to suggest that HMGR inhibition causes a reduction in available MVA for FDP synthesis leading to decreased protein prenylation and, consequently, limited cell division. The addition of exogenous FDP reverses this affect by satisfying Ftase substrate demand.

The third possible reason for the FDP-induced elevated growth response may be that factors limiting cell division are derived from FDP. In the cultures used, CK were not limiting since the media contained iP. Therefore, CK-regulated complexes in the cell division cycle were activated. Responses of cells to the addition of different concentrations of mevastatin, however, revealed a "transient" reduction in cell division before recovery. If iP was present, what caused this reduction in cell growth? Sterols are derivatives of FDP, synthesized from the C_{20} molecule squalene. Cell division activity requires a significant level of sterol biosynthesis (Chappell, 1995). Narita and Gruissem (1989) found that early fruit development in tomato was sensitive to mevinolin (a mevastatin analogue) but carotenoid biosynthesis continued. In explaining this, they suggested that HMGR activity might be limiting only for sterol biosynthesis which would result in less cell division and reduced fruit development. Furthermore, Cowan *et al.* (1997) showed that mevastatin-treated avocado fruit accumulated ABA. It is well established that lipid membrane integrity is disrupted by high levels of ABA and that phytosterols inhibit this action (Stillwell *et al.*, 1990). While ABA content was not monitored following mevastatin addition in avocado cell cultures, it could be postulated that a rise in ABA with a concomitant reduction in sterol biosynthesis due to

the inhibition of HMGR, would disrupt membrane integrity and result in decreased cell division and even cell death. The reestablishment of growth after addition of FDP to mevastatin-treated cells could be explained in part, by a restoration of sterol biosynthesis and membrane integrity. The partial response of arrested cell cultures to stigmasterol alone, however, suggests that a combination of the first hypothesis (variable HMGR isozymes dedicated to the production of specific classes of isoprenoids) and the second hypothesis (reduced protein prenylation) is more likely.

In order to put together a more comprehensive model of molecular interactions with the cell cycle in cultured cells, two more points need to be mentioned. The first is with regard to ABA. In addition to its effects on membrane integrity, ABA has been implicated in the direct inhibition of cell division by blocking cellular nucleic acid and protein synthesis (Meyers *et al.*, 1990). Further, increased ABA levels have been correlated with the inhibition of cytosolic HMGR activity in pea seedlings (Russell and Davidson, 1982) and maize endosperm tissue (Meyers *et al.*, 1990). Thus, the increase in ABA due to HMGR inhibition (Cowan *et al.*, 1997) may play a central role in mevastatin-induced cessation of cell growth. The second interaction involves sucrose. In *in vitro* studies, cultured tissue requires a carbon source which is commonly supplied in the form of sucrose. Sucrose has recently been implicated in mitotic induction and protein synthesis (Koch, 1996) and it has been shown to induce a G_1 cyclin gene coding for the δ2-cyclin protein in *Arabidopsis* (Taiz and Zeiger, 1998). In cultured cells then, the synergistic action of AUX, CK and sucrose leads to the activation of a CDK-G_1 cyclin complex which triggers the entry of cells into the S phase of the cell cycle. The effects of HMGR-inhibition act antagonistically to these systems and result in the cessation of cell division. The interactions discussed are summarised in Fig. 5.2. Further elucidation of the spatial and temporal regulation of the components involved in this system may shed some light on the developmental processes involved in fruit development and the physiological problems associated with this program.

5.2 CONCLUSIONS AND FUTURE PROSPECTS

This study has demonstrated that cell cultures, initiated from 'Hass' avocado seed tissue, can be maintained and used as a model system for metabolic studies. Further, cell culture responses closely resemble *in vivo* responses to induced stress scenarios. This property makes this system extremely valuable for use in investigating practical research problems. This study also demonstrated that a highly complex system, operating at the level of gene transcription, is involved in isoprenoid modulation of cell division.

Immediate research priorities should focus on:

1. Developing a reproducible system for isolating protoplasts from mesocarp tissue. The potential problem area was identified as the cellulase source and it would be a relatively simple procedure to test this hypothesis. The implications of the success of this are far reaching and immediate application could be found in generating a similar procedure for cell suspensions. Subsequent genetic modification would not be too far in the future.
2. Optimising growth conditions of cell cultures in terms of inorganic and organic nutrient requirements and the physical environment for rapid growth.
3. In terms of the isoprenoid-cell division regulation model, research should focus on the HMGR isozyme and compartmentalized pathway theory proposed by Chappell *et al.* (1995). The identification of HMGR isoforms and the associated pathways would provide much needed answers to apparent anomalies in the present research model.

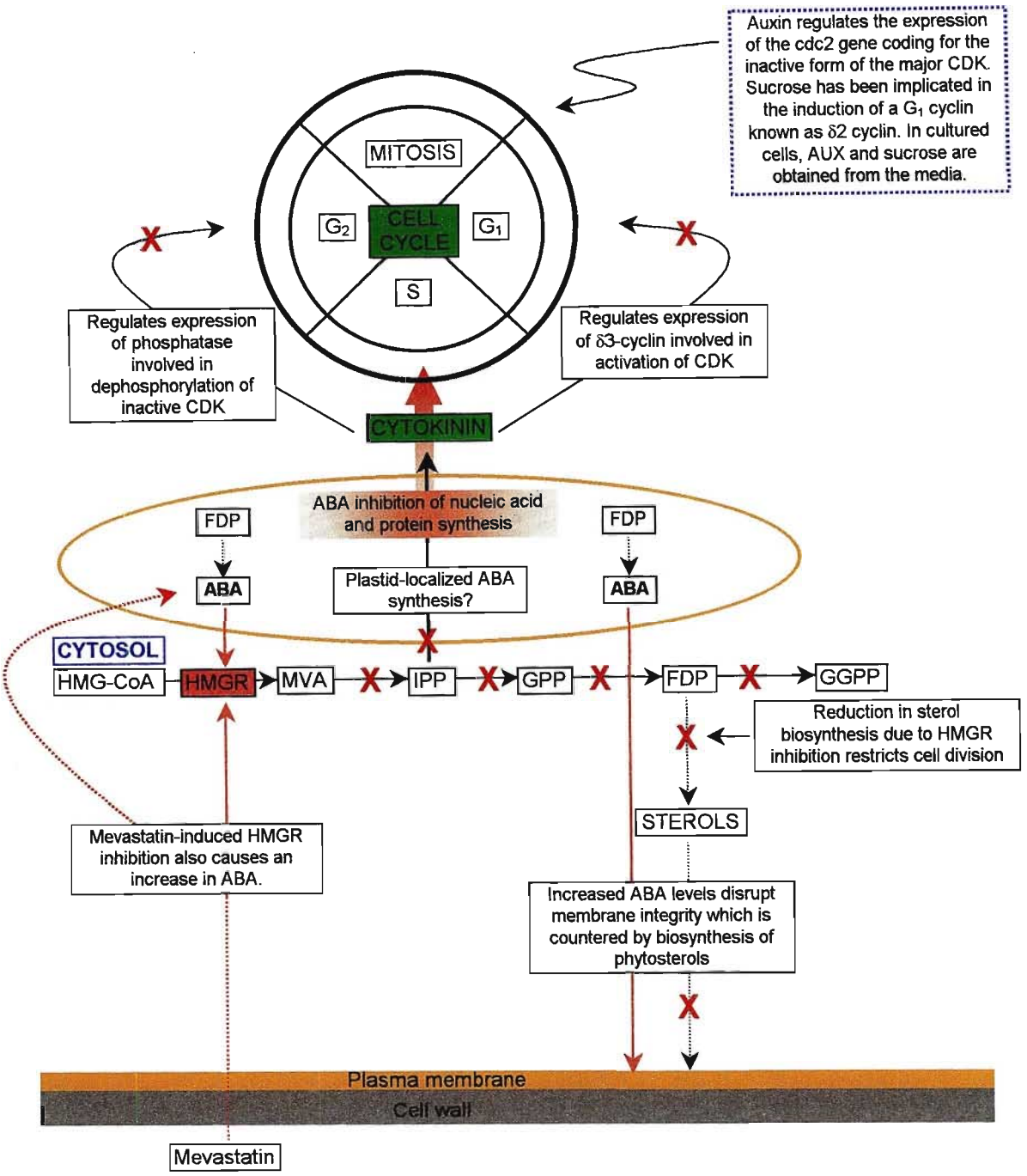


Figure 5.2

Proposed interactions of ABA, CK and sterols in response to HMGR inhibition by mevastatin in cultured avocado cells. Pathways marked with a cross do not function when HMGR activity is blocked. There is a growing body of evidence that suggests specific isoprenoid pathways are mediated by different isozymes of HMGR and that compartmentalization of these processes, separates products and their biosynthetic enzymes. Evidence for the existence of these "discrete channels" could explain the apparent anomalies such as increased ABA synthesis in response to HMGR inhibition and the reversal of mevastatin-induced HMGR inhibition by FDP.

LITERATURE CITED

Ahmed, M. F., A. S. Kantharajah and P. Holford. 1998. *In vitro* regeneration of avocado (*Persea americana* Mill.) and transient gene expression. *Acta Hort.* 461: 339-347

Allan, E. 1991. Plant cell culture. In: Stafford, A and G. Warren (Eds.) Plant cell and tissue culture. Open University Press, Buckingham

Assman, G. and H. Schriever. 1980. Role of low density and high density lipoproteins in artherogenesis. In: Fondu, M. (Ed.) Lipids and lipoproteins. *Proc. of a Scientific Symposium on Int. Fed. Of Margarine Assn.* Brussels

Awad, M. and R. E. Young. 1979. Postharvest variation in cellulase, polygalacturonase and pectinmethylesterase in avocado (*Persea americana* Mill. cv. Fuerte) fruits in relation to respiration and ethylene production. *Plant Physiol.* 64: 306-308

Bach, T. J. 1987. Synthesis and metabolism of mevalonic acid in plants. *Plant Physiol. Biochem.* 25: 163-178

Bach, T. J. and H. K. Lichtenthaler. 1983. Inhibition by mevinolin of plant growth, sterol formation and pigment accumulation. *Physiol. Plant* 59: 50-60

Behnke, M. 1979. Selection of potato callus for resistance to culture filtrates of *Phytophthora infestans* and regeneration of resistant plants. *Theor. Appl. Genet.* 55: 69-71

Behnke, M. 1980. General resistance to blight of *Solanum tuberosum* plants regenerated from callus resistant to culture filtrates of *Phytophthera infestans. Theor. Appl. Genet.* 56: 151-152

Bengochea, T. and J. H. Dodds. 1986. Plant protoplasts: a biotechnological tool for plant improvement. Chapman and Hall, London

Ben-Ya'acov, A. and E. Michelson. 1995. Avocado rootstocks. *Hort. Rev.* 17: 381-429

Bergh, B. O. 1976. Avocado breeding and selection. In: Sauls, J. W., R. L. Phillips and L. K. Jackson (Eds.). *Proc. 1st Internat. Trop. Fruit Short Course: The avocado.* Univ. of Fla., Gainsville, Florida

Bergh, B. O. 1987. Avocado breeding in California. *S. Afr. Avocado Growers Assoc. Yearb.* 10: 22-23

Bergh, B. O. 1991a. The avocado and human nutrition. I. Some human health aspects of the avocado. *Proc. of Second World Avocado Congr.* 1: 25-35

Bergh, B. O. 1991b. The avocado and human nutrition. II. Avocados and your heart. *Proc. of Second World Avocado Congr.* 1: 37-47

Bergh, B. and N. Ellstrand.1986. Taxonomy of avocado. *Calif. Avocado Soc. Yearb* 70: 127-133

Biale, J. B and R. E. Young. 1971. The avocado pear. In: Hulme, A. C. (Ed.) The biochemistry of fruit and their products. Vol. 2. Academic Press, NY pp. 1-63

Bijzet, Z., A. D. Sippel, B. Snijder, H. J. Breedt and B. Cilliers. 1995. An overview of avocado breeding and evaluation of genotypes in the Republic of South Africa. *Proc. of Third World Avocado Congr.* 119-123

Blickle, W., H. P. Muhlbach and H. L. Sanger. 1986. Conditions for the isolation of protoplasts from callus cultures of avocado (*Persea americana* Mill.). *6th International Congress of Plant Tissue Culture.* Minneapolis pp. 357 (Abstract)

Blumenfeld, A. and S. Gazit. 1971. Growth of avocado fruit callus and its relation to exogenous and endogenous cytokinin. *Physiol. Plant.* 25: 369-371

Bornman, C. H. 1987. *Picea abies.* In: Bonga, J. M. and D. J. Durzan (Eds.) Cell and Tissue Culture in Forestry Vol. 3. Martinus Nijhoff, Dordrecht pp. 2-29

Bower, J. P. and J. G. Cutting. 1988. Avocado fruit development and ripening physiology. *Hort. Rev.* 10: 229-271

Caponetti, J. D. 1996. Nutrition of callus cultures. In: Trigiano, R. N. and D. J. Gray (Eds.) Plant tissue culture concepts and laboratory exercises. CRC Press, New York

Chappell, J. 1995. The biochemistry and molecular biology of isoprenoid metabolism. *Plant Physiol.* 107: 1-6

Chappell, J., F. Wolf, J. Proulx, R. Cuellar and C. Saunders. 1995. Is the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase a rate-limiting step for isoprenoid biosynthesis in plants? *Plant Physiol.* 109: 1337-1343

Choi, D., B. L. Ward and R. M. Bostock. 1992. Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to *Phytophthera infestans* and to its elicitor arachidonic acid. *The Plant Cell* 4: 1333-1344

Coffey, M. D. 1992. Phytophthora root rot of avocado. In: Kumar, J., H. S. Chaube, V. S. Singh and A. N. Mukhopadhyay (Eds.) Plant diseases of international importance Vol. 3. Prentice Hall, Englewood Cliffs, NJ pp. 423-444

Collin, H. A. and S. Edwards. 1998. Plant cell culture. Bios Scientific Publishers, UK

Constable, F. Isolation and culture of plant protoplasts. In: Wetter, L. R. and F. Constable (Eds.) Plant tissue culture methods. National Research Council of Canada, Saskatoon pp. 38-48

Coombe, B. G. 1976. The development of fleshy fruit. *Ann. Rev. Plant Physiol.* 37: 377-405

Cowan, A. K., C. S. Moore-Gordon, I. Bertling and B. N. Wolstenholme. 1997. Metabolic control of avocado fruit growth. *Plant Physiol.* 114: 511-518

Crowell, D. N. and M. S. Salaz. 1992. Inhibition of cultured tobacco cells at low concentrations of lovastatin is reversed by cytokinin. *Plant Physiol.* 100: 2090-2095

Davis, J., D. Henderson, M. Kobayashi, M. T. Clegg and M. T. Clegg. 1998. Genealogical relationships among cultivated avocado as revealed through RFLP analyses. *Amer. Genetic Assoc.* 89: 319-323

Delanney, X., B. J. LaVallee, R. K. Proksch, R. L. Fuchs, S. R. Sims, J. T. Greenplate, P. G. Marrone, R. B. Dodson, J. J. Augustine, J. G. Layton and D. A. Fischhoff. 1989. Field performance of transgenic tomato plants expressing the *Bacillus thuringiensis* var. Kurstaki insect control protein. *Bio/Technology* 7: 1265-1269

Desjardins, P. R. 1958. Callus tissue growth on avocado stem segments cultured on artificial media. *Calif. Avocado Soc. Yearb.* 42: 99-101

Dhir, S. K., S. Dhir and J. M. Widholm. 1991. Plantlet regeneration from immature cotyledon protoplasts of soybean (*Glycine max* L.). *Plant Cell Rep.* 10: 39-43

Dickinson, C. D., T. Altabella and M. J. Chrispeels. 1991. Slow-growth phenotype of transgenic tomato expressing apoplastic invertase. *Plant Physiol.* 95: 420-425

Dixon, R. A. and K. W. Fuller. 1976. Effects of synthetic auxin levels on phaseolin production and phenylalanine ammonialyase (PAL) activity in tissue cultures of *Phaseolus vulgaris* L. *Physiol. Plant Pathol.* 11: 287-296

Eriksson, T. R. 1985. Protoplast isolation and culture. In: Fowke, L. and F. Constable (Eds.) Plant protoplasts. CRC Press, Florida

Evans, H. M. and K. S. Bishop. 1922. On the existence of a hitherto unrecognised dietary factor essential for reproduction. *Science* 56: 650-651

Fitzsimons, P. J. and J. D. B. Weyers. 1985. Properties of some enzymes used for protoplast isolation. In: Pilet, P. E. (Ed.) The physiological properties of plant protoplasts. Springer-Verlag, Berlin

Fukuzawa, K., A. Tokumura, S. Ouchi and H. Tsukatani. 1982. Antioxidant activities of tocopherols on Fe^{2+}-ascorbate-induced lipid peroxidation in lectithin liposomes. *Lipid* 17: 511-513

Gaillard, J. P. and J. Godefroy. 1995. Avocado. Macmillan, London

Gamborg, O. L., R. A. Miller and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50: 151-158

Gazit, S. and A. Blumenfeld. 1970. Cytokinin and inhibitor activities in the avocado fruit mesocarp. *Plant Physiol.* 46: 334-336

Gazit, S. and A. Blumenfeld. 1971. Tissue cultures of callus derived from avocado fruit. *Calif. Avocado Soc. Yearb.* 55: 105-109

Gillaspy, G., H. Ben-David and W. Gruissem. 1993. Fruits: a developmental perspective. *Plant Cell* 5: 1439-1451

Gmitter, F. G. Jr., J. W. Grosser and G. A. Moore. 1992. Citrus. In: Hammerschlag, F. A. and R. E. Litz (Eds.) Biotechnology of perennial fruit crops. CAB International, Wallingford, UK pp. 335-369

Gonzalez-Rosas, H. and S. Salazar-Garcia. 1984. Root induction and vegetative development from avocado plantlets (*Persea americana* Mill.). *Calif. Avocado Soc. Yearb.* 68: 167-171

Gonzalez-Rosas, H., S. Salazar-Garcia and V. Vazquez Valdivia. 1985. Propagation *in vitro* of chinini (*Persea schiedeana* Ness.). *Calif. Avocado Soc. Yearb.* 69: 125-131

Gonzalez-Rosas, H., B. E. Llano-Agudelo and S. Salazar-Garcia. 1990. Effect of IBA, kinetin and benzil amino purine on the germination, shoot development and root formation in avocado embryos cultivated *in vitro*. *Calif. Avocado Soc. Yearb.* 75: 201-205

Gould, A. R. 1984. Control of the cell cycle in cultured plant cells. *CRC Crit. Rev. Plant Sci.* 1(4): 315-344

Gray, J. C. 1987. The control of isoprenoid biosynthesis in higher plants. *Adv. Bot. Res.* 14: 25-91

Grosser, J. W. 1994. Observations and suggestions for improving somatic hybridization by plant protoplast isolation, fusion and culture. *HortScience* 29(11): 1241-1242

Grosser, J. W. and J. L. Chandler. 1987. Asceptic isolation of leaf protoplasts from *Citrus*, *Poncirus*, *Citrus* x *Poncirus* hybrids and *Severinia* for use in somatic hybridisation experiments. *Scientia Hort.* 31: 253-257

Grosser, J. W. and F. G. Gmitter Jr. 1990. Protoplast fusion and Citrus improvement. *Plant Breeding Rev.* 8: 339-374

Grusak, M. A. and D. Dellapenna. 1999. Improving the nutrient composition of plants to enhance human nutrition and health. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 50: 133-161

Hain, R., B. Bieseler, H. Kindl, G. Schroeder and R. Stocker. 1990. Expression of a stilbene synthase gene in *Nicotiana tabacum* results in synthesis of the phytoalexin resveratol. *Plant Molecular Biology* 15: 325-335

Hammerschlag, F. A. 1988. Selection of peach cells for insensitivity to culture filtrates of *Xanthomonas campestris* pv. *pruni* and regeneration of resistant plants. *Theor. Appl. Genet.* 76: 865-869

Hartmann, M. A. and P. Benveniste. 1987. Plant membrane sterols: isolation, identification and biosynthesis. *Methods Enzymol.* 148: 632-650

Hatfield, R. and D. J. Nevins. 1986. Characterisation of the hydrolytic activity of avocado cellulase. *Plant Cell Physiol.* 27: 541-552

Hemerly, A. S., P. C. G. Ferreira, M. van Montagu and D. Inze. 1999. Cell cycle control and plant morphogenesis: is there an essential link? *BioEssays* 21: 29-37

Hooley, R. 1982. Protoplasts isolated from aleurone layers of wild oat (*Avena fatua* L.) exhibit the classic response to gibberellic acid. *Planta* 154: 29-40

Hughes, B. G., F. G. White and M. A. Smith. 1976. Scanning electron microscopy of barley protoplasts. *Protoplasma* 90: 399-405

Human, T. P. 1987. Oil as a byproduct of the avocado. *S. Afr. Avocado Growers Yearb.* 10: 159-162

Jacobs, T. W. 1995. Cell cycle control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 46: 317-319

Jain, R. K., S. Jain and J. B. Chowdhury. 1991. In vitro selection for salt tolerance in *Brassica juncea* L. using cotyledon explants, callus and cell suspension cultures. *Ann. Bot.* 67: 517-519

Kao, K. N. and M. R. Michayluk. 1975. Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. *Planta* 126: 105-110

Kay, E. and C. A. Schroeder. 1963. Seasonal regeneration of avocado fruit tissue *in vitro*. *J. Amer. Soc. Hort. Sci.* 83: 287-290

Klann, E. M., B. Hall and A. B. Bennett. 1996. Antisense acid invertase (TIV1) gene altars soluble sugar compostion and size in transgenic tomato fruit. *Plant Physiol.* 112: 1321-1330

Kleinig, H. 1989. The role of plastids in isoprenoid biosynthesis. *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* 40: 39-59

Knight, R. J. and H. F. Winters. 1971. Mango and avocado selection in southeastern Florida. *Proc. Flor. St. Hort. Soc.* 84: 314-317

Koch, K. E. 1996. Carbohydrate-modulated gene expression. *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* 47: 509-540

Lahav, E., U. Lavi, C. Degani and S. Gazit. 1995. Avocado breeding in Israel. *Proc. of Third World Avocado Congr.* 106-113

Larkin, P. J. 1976. Purification and viability determinations of plant protoplasts. *Planta* 128: 213-216

Lavi, U., E. Lahav, A. Genizi, C. Degani, S. Gazit and J. Hillel. 1991. Quantitative genetic analysis of traits in avocado cultivars. *Plant Breeding* 106: 149-160

Lavi, U., E. Lahav, C. Degani and S. Gazit. 1992. The genetics of the juvenile phase in avocado and its application for breeding. *J. Amer. Soc. Hort. Sci.* 117(6): 981-984

Lavi, U., E. Lahav, C. Degani, S. Gazit and J. Hillel. 1993. Genetic variance components and heritabilities of several avocado traits. *J. Amer. Soc. Hort. Sci.* 118(3): 400-404

Lindsey, K. and M. M. Yeoman. 1985. Dynamics of plant cell cultures. In: Vasil, I. K. (Ed.) Cell culture and somatic cell genetics of plants. Vol. 2. Academic Press, London

Litz, R. E. and D. J. Gray. 1992. Organogenesis and somatic embryogenesis. In: Hammerschlag, F. A. and R. E. Litz (Eds.) Biotechnology of Perennial Fruit Crops. CAB International, Wallingford, UK pp. 3-34

Lloyd, G. and B. McCowan. 1981. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. *Intl. Plant Prop. Soc. Proc.* 30: 421-427

Lorz, H., E. Gobel and P. Brown. 1988. Advances in tissue culture and progress towards genetic transformation of cereals. *Plant Breed.* 100: 1-25

Maluszynski, M., B. S. Aloowalia and B. Sigurbjornsson. 1995. Application of *in vivo* and *in vitro* mutation techniques for crop improvement. *Euphytica* 85: 303-315

Meyers, P. N., T. L. Setter, J. T. Madison and J. F. Thompson. 1990. Abscisic acid inhibition of endosperm cell division in cultured maize kernels. *Plant Physiol.* 94: 1330-1336

Mohamed-Yasseen, Y. 1992. *In vitro* propagation of avocado (*Persea americana* Mill.). *Calif. Avocado Soc. Yearb.* 77: 107-111

Mohamed-Yasseen, Y., R. Schnell, R. Knight and T. L. Davenport. 1992. *In vitro* plant regeneration from avocado (*Persea americana* Mill.). *HortScience* 27: 696 (Abstract)

Mooney, P. A. and J. Van Staden. 1987. Induction of embryogenesis in callus from immature embryos of *Persea americana*. *Can. J. Bot.* 65: 622-626

Moore, K. B. and K. K. Oishi. 1994. 3-hydroxy-3-methylglutaryl CoA reductase activity in the endosperm of maize vivipary mutants. *Plant Physiol.* 105: 119-125

Moore-Gordon, C. S., A. K. Cowan, I. Bertling, C. E. J. Botha and R. H. M. Cross. 1998. Symplastic solute transport and avocado fruit development: a decline in cytokinin/ABA ratio is related to the appearance of the Hass small fruit variant. *Plant Cell Physiol.* 39(10): 1027-1038

Morehead, T. A., B. J. Biermann, D. N. Crowell and S. K. Randall. 1995. Changes in protein isoprenylation during the growth of suspension-cultured tobacco cells. *Plant Physiol.* 109: 277-284

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497

Nakasone, H. Y. and R. E. Paull. 1998. Avocado. In: Tropical Fruits. CAB International, New York. pp 76-102

Narita, J. O. and W. Gruissem. 1989. Tomato hydroxymethylglutaryl-CoA reductase is required early in fruit development but not during ripening. *Plant Cell* 1: 181-190

Nel, D. D., J. M. Kotze and C. P. Snyman. 1982. *In vitro* propagation of *Persea indica*. *Calif. Avocado Soc. Yearb.* 66: 167-168

Ochatt, S. J. 1990. Protoplast technology for top-fruit tree breeding. *Acta Hort.* 280: 215-226

Ochatt, S. J. and E. M. Patat-Ochatt. 1995. Protoplast technology for the breeding of top-fruit trees (*Prunus*, *Pyrus*, *Malus*, *Rubus*) and woody ornamentals. *Euphytica* 85: 287-294

Ochatt, S. J. and J. B. Power. 1988. Plant regeneration from leaf mesophyll protoplasts of Williams' Bon Chretien (Syn. Bartlett) pear (*Pyrus communis* L.). *Plant Cell Rep.* 7: 587-589

Ochatt, S. J. and J. B. Power. 1992. Plant regeneration from protoplasts of higher plants. In: Fowler, M. W., G. S. Warren and M. Moo-Young (Eds.) Comprehensive Biotechnology Second Supplement. Pergamon Press, New York

Ochatt, S. J., E. M. Patat-Ochatt and J. B. Power. 1992. Protoplasts. In: Hammerschlag, F. A. and R. E. Litz (Eds.) Biotechnology of Perennial Fruit Crops. CAB International, Oxford

Patat-Ochatt, E. M., S. J. Ochatt and J. B. Power. 1988. Plant regeneration from protoplasts of apple rootstocks and scion varieties (*Malus* x *domesticus* Borkh). *J. Plant Physiol.* 133: 460-465

Percival, F. W., L. G. Cass, K. R. Bozak and R. E. Christoffersen. 1991. Avocado fruit protoplasts: a cellular model system for ripening studies. *Plant Cell Reports* 10: 512-516

Piqueras, A., E. Olmas and E. Hellin. 1994. Cytological changes related to salt tolerance in embryogenic callus of *Citrus limon*. *Plant Cell, Tissue and Organ Culture* 39: 13-18

Platt-Aloia, K. A., W. W. Thomson and R. E. Young. 1980. Ultrastructural changes in the walls of ripening avocados: transmission, scanning and freeze fracture microscopy. *Bot. Gaz.* 141(4): 366-373

Platt-Aloia, K. A., J. W. Oross and W. W. Thomson. 1983. Ultrastructural study of the development of oil cells in the mesocarp of avocado fruit. *Bot. Gaz.* 144(1): 49-55

Pliego-Alfaro, F. 1988. Development of an *in vitro* rooting bioassay using juvenile-phase stem cuttings of *Persea americana* Mill. *J. Hort. Science* 63(3): 295-301

Pliego-Alfaro, F. and B. O. Bergh. 1992. Avocado. In: Hammerschlag, F. A. and R. E. Litz (Eds.). Biotechnology of Perennial Fruit Crops. CAB International, Wallingford, UK pp. 323-333

Pliego-Alfaro, F. and T. Murashige. 1987. Possible rejuvenation of adult avocado by grafting onto juvenile rootstocks *in vitro*. *HortScience* 22: 1321-1324

Pliego-Alfaro, F. and T Murashige. 1988. Somatic embryogenesis in avocado (*Persea americana* Mill.) *in vitro*. *Plant Cell, Tissue and Organ Culture* 12: 61-66

Power, J. B., J. V. Chapman and D. Wilson. 1984. Laboratory manual: plant tissue culture. University of Nottingham, Nottingham

Qian, D., D. Zhou, R. Ju, C. L. Cramer and Z. Yang. 1996. Protein farnesyltransferase in plants: molecular characterization and involvement in cell cycle control. *Plant Cell* 8: 2381-2394

Raghaven, V. 1976. Experimental embryogenesis in vascular plants. Academic Press, New York

Randall, S. K., M. S. Marshall and D. N. Crowell. 1993. Protein isoprenylation in suspension cultured tobacco cells. *Plant Cell* 5: 433-442

Richings, E. W., R. F. Cripps and A. K. Cowan. 2000. Factors affecting 'Hass' avocado fruit growth: sugar, isoprenoid and abscisic acid metabolism. *Physiol. Plant.* (in press)

Rodriguez-Concepcion, M., S. Yaluvsky and W. Gruissem. 1999. Protein prenylation in plants: old friends and new targets. *Plant Mol. Biol.* 39: 865-870

Rotman, B. and B. W. Papermaster. 1966. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. *Proc. Natl. Acad. Sci.* 55: 134-141

Russell, D. W. and H. Davidson. 1982. Regulation of cytosolic HMG-CoA reductase activity in pea seedlings: contrasting responses to different hormones, and hormone-product interaction suggest hormonal modulation of activity. *Biochem. Biophys. Res. Commun.* 104: 1537-1543

Sahar, N. and P. Spiegel-Roy. 1984. *In vitro* germination of avocado. *HortScience* 19: 886-888

Schmitt, D., K. Callan and W. Gruissem. 1996. Molecular and biochemical characterization of tomato farnesyl-protein transferase. *Plant Physiol.* 112: 767-777

Schopke, C., L. E. Muller and H. W. Kohlenbach. 1987. Somatic embryogenesis and regeneration of plantlets in protoplast cultures from somatic embryos of coffee (*Coffea canephora* P. ex Fr.). *Plant, Cell, Tissue and Organ Culture* 8: 243-248

Schroeder, C. A. 1953. Growth and development of the 'Fuerte' avocado fruit. *Proc. Amer. Soc. Hort. Sci.* 68: 253-258

Schroeder, C. A. 1955. Proliferation of mature fruit pericarp tissue slices *in vitro*. *Science* 122: 601

Schroeder, C. A. 1956. Growth of avocado fruit tissue on artificial media. *Calif. Avocado Soc. Yearb.* 40: 165-168

Schroeder, C. A. 1961. Some morphological aspects of fruit tissues grown *in vitro*. *Bot. Gaz.* 112: 198-204

Schroeder, C. A. 1963. Induced temperature tolerance of plant tissue *in vitro*. *Nature* 200: 1301-1302

Schroeder, C. A. 1967. Fruit tissue culture with special reference to plant growth regulators. *Proc. Intl. Symp. On Subtropical and Tropical Horticulture* pp. 191-200

Schroeder, C. A. 1968. The longevity of avocado tissue *in vitro*. *Calif. Avocado Soc. Yearb.* 52: 128-130

Schroeder, C. A. 1971. The response of avocado pericarp tissue to temperature and light *in vitro*. *Calif. Avocado Soc. Yearb.* 54: 85-89

Schroeder, C. A. 1973. The response of apical meristem and other tissues of avocado in aseptic culture. *Calif. Avocado Soc. Yearb.* 56: 138-141

Schroeder, C. A. 1975. Response of avocado flower buds and floral parts cultured *in vitro*. *Calif. Avocado Soc. Yearb.* 58: 66-73

Schroeder, C. A. 1976. Responses of avocado stem pieces in tissue culture. *Calif. Avocado Soc. Yearb.* 60: 160-163

Schroeder, C. A. 1977. Longevity of plant tissue cultures. *Calif. Avocado Soc. Yearb.* 61: 72-74

Schroeder, C. A. 1979. Etiolation and avocado bud elongation *in vitro*. *Calif. Avocado Soc. Yearb.* 63: 86-89

Schroeder, C. A. 1980. Avocado tissue *in vitro*. *Calif. Avocado Soc. Yearb.* 64: 139-141

Schroeder, C. A. and E. Kay. 1961. Temperature condition and tolerance of avocado fruit tissue. *Calif. Avocado Soc. Yearb.* 45: 87-92

Schroeder, C. A., E. Kay and L. H. Davis. 1962. Totipotency of cells from fruit pericarp tissue *in vitro*. *Science* 138: 595-596

Scora, R. W. and B. Bergh. 1990. The origins and taxonomy of avocado (*Persea americana* Mill.) Lauraceae. *Acta Hortic.* 275: 387-394

Scowcroft, W. R. 1985. Somaclonal variation: the myth of clonal uniformity. In: Hohn, B. and E. S. Dennis (Eds.) Genetic flux in plants. Springer-Verlag, Wien, pp. 217-245

Seymour, G. B. and G. A. Tucker. 1993. Avocado. In: Seymour, G. B., J. E. Taylor and G. A. Tucker (Eds.) Biochemistry of fruit ripening. Chapman and Hall, London pp. 53-81

Skene, K. M. and M. Barlass. 1983. *In vitro* culture of abscised immature avocado embryos. *Ann. Bot.* 52: 667-672

Skirvin, R. M., K. D. McPheeters and M. Norton. 1994. Sources and frequency of somaclonal variation. *HortScience* 29(11): 1232-1237

Slor, E. and R. Spodheim. 1972. Selection of avocado varieties in Israel. *Calif. Avocado Soc. Yearb.* 55: 156-157

Smith, M. A. L. 1994. Plant Tissue Culture. In: *Encyclopedia of Agricultural Sciences*, Vol. 3. Academic Press, N. Y. pp. 369-384

Smith, M. K. and R. A. Drew. 1990. Current applications of plant tissue culture in plant propagation and improvement. *Aust. J. Plant Physiol.* 17: 267-289

Solorzano-Vega, D. E. 1989. Propagation *in vitro* of rootstocks of avocado. *Calif. Avocado Soc. Yearb.* 73: 149-151

Soni, R., J. P. Carmichael, Z. H. Shah and J. A. H. Murray. 1995. A family of cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. *Plant Cell* 7: 85-103

Stillwell, W., B. Bringle, P. Hester and S. R. Wassall. 1989. Interaction of abscisic acid with phospholipid membranes. *Biochemistry* 28: 2798-2804

Stillwell, W., Y. F. Cheng and S. R. Wassall. 1990. Plant sterol inhibition of abscisic acid-induced perturbations in phospholipid bilayers. *Biochem. Biophys. Acta* 1024: 345-351

Storey, W. B., B. Bergh and G. A. Zentmyer.1986. the origin, indigenous range and dissemination of the avocado. *Calif. Avocado Soc. Yearb.* 70: 127-133

Taiz, L. and E. Zeiger. 1998. Plant physiology. Sinauer Associates, Masachusetts

Takebe, I., G. Labib and G. Melchers. 1971. Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. *Naturwissenschaften* 58: 318-320

Tisserat, B., E. B. Esan and T. Murashige. 1979. Somatic embryogenesis in angiosperms. *Hort. Rev.* 1: 3-78

Vaeck, M., A. Reynaerts, H. Hofte, S. Jansens, M. De Beuckaleer, C. Dean, M. Zabeau, M. Van Montagu and J. Leemans. 1987. Transgenic plants protected from insect attack. *Nature* 328: 33-37

Van Lelyveld, L. J. 1984. Summary of a report on an investigation into physiological disorders of avocados. *Calif. Avocado Soc. Yearb.* 68: 183-185

Vardi, A., P. Spiegel-Roy and E. Galvin. 1975. Citrus cell culture: isolation of protoplasts, plating densities, effects of mutagens and regeneration of embryos. *Plant Sci. Lett.* 4: 231-236

Whiley, A. W. 1994. Ecophysiological studies and tree manipulation for maximisation of yield potential in avocado (*Persea americana* Mill.). Unpubl. PhD Thesis, Dept. Hort. Sci., Univ. Natal, South Africa

Whiley, A. W. and B. Schaffer. 1994. Avocado. In: Schaffer, B. and P. C. Anderson (eds.). CRC Handbook of environmental physiology of fruit crops. Vol. 2, Subtropical and tropical crops. CRC Press Inc., Boca Raton pp 3-35

Whiley, A. W., J. S. Kohne, M. L. Arpaia and G. S. Bender. 1990. Future prospects with new avocado cultivars and elite rootstocks. *S. Afr. Avocado Grower's Assoc. Yearb.* 13: 16-20

Williams, E. G., I. M. Verry and W. M. Williams. 1982. Use of embryo culture in interspecific hybridization. In: Vasil, I. K., W. R. Scowcroft and K. J. Frey (Eds.) Plant improvement and somatic cell genetics. Academic Press, New York pp. 119-128

Williams, L. O. 1976. The botany of the avocado and its relatives. In: Sauls, J. W., R. L. Phillips and L. K. Jackson (Eds.). *Proc. 1st Internat. Trop. Fruit Short Course: The avocado.* Univ. of Fla., Gainsville

Wolstenholme, B. N. and A. W. Whiley. 1995. Strategies for maximising avocado productivity: an overview. *Proc. of Third World Avocado Congr.* 61-70

Yalovsky, S., A. E. Lorraine and W. Gruissem. 1996. Specific prenylation of tomato Rab proteins by geranylgeranyl type-II transferase requires a conserved cysteine-cysteine motif. *Plant Physiol.* 110: 1349-1359

Yasuda, T., M. Tahara, N. Uchida and T. Yamaguchi. 1986. Somatic embryogenesis from coffee callus and protoplasts. *VI International Congress of Plant Tissue and Cell Culture*, Minneapolis, pp. 137 (Abstract)

Young, M. J. 1983. Avocado callus and bud culture. *Proc. Fla. St. Hort. Soc.* 96: 181-182

van Zyl, J. L. and S. G. Ferreira. 1995. An overview of the avocado industry in South Africa. *S. Afr. Avocado Growers Assoc. Yearb.* 18: 23-30

Zapata, F. J., M. S. Alejar, L. B. Torrizo, A. V. Novero, V. P. Singh and D. Senadhira. 1991. Field performance of anther culture-derived lines from F_1 crosses of Indica rices under saline and non-saline conditions. *Theor. Appl. Genet.* 83: 6-11

Zhang, K., D. S. Letham and P. C. L. John. 1996. Cytokinin controls the cell cycle at mitosis by stimulating the tyrosine dephosphorylation and activation of p34cdc2-like H1 histone kinase. *Planta* 200: 2-12

Zilkah, S. and I. Klein. 1987. Growth kinetics and determination of shape and size of small and large fruits cultivar Hass on the tree. *Scientia Hort.* 32: 195-202