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Asparagus somatic embryogenesis: detection of somaclonal variation using molecular and cytological analyses

A thesis presented in partial fulfilment of the requirements for the degree

of

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Wendy Hollingsworth

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Dedicated to:

My parents - Rudolph and Shirley Hollingsworth

"...............because you love me.............."

In pursuit of excellence !!!!!!

Abstract

The embryogenic potential for six asparagus cultivars (Aspiring, Karapiro, Pacifica, Turoa, Syn4, and UC157), and the genetic stability of the somatic embryogenic system were investigated. Experiments 1 to 3 investigated the embryogenic potential of select cultivars, whereas experiments 4 to 7 analysed the genetic stability of embryogenic cells and plantlets. In experiment 8, morphological, anatomical, cytological and molecular techniques were used to characterise different types of calli identified during the study.

For all cultivars, embryogenic callus was promoted on Murashige and Skoog (MS) media containing 3% sucrose, 1% agar and one of the following plant growth regulator (PGR) concentrations: 0.3, 1, 3, and 10 µM 2,4-D and 1.0 µM NAA/ 0.1 µM Kinetin. Plant genotype, PGR concentration and length of time in culture significantly influenced both the number of explants producing calli and the type of calli developing from explants.

The following sequence was found to be most effective in producing complete plantlets from embryogenic calli: callus induction (CI) on Murashige and Skoog (MS) media containing 3% sucrose, 1% agar and either of 1.0, 3.0 and 10 µM 2,4-D, followed by transfer onto liquid embryo induction media (EI) containing MS + 6% sucrose and finally regeneration on regeneration media (Rg4) containing MS + 0.2 g/l glutamine + 3% sucrose + 1% agar. Treatment of 'Pacifica' globular embryos at -15 ^{0}C for 3 hr produced the highest percent converted plantlets (34 and 26% for 6-month-old embryogenic calli and 1 year-old embryogenic suspension cells respectively).

The number of *in vitro*-regenerated asparagus plantlets surviving acclimatisation was increased by acclimatising plantlets with minicrowns that contain 2-5 storage roots, and by removal of *in vitro*-formed cladophylls prior to acclimatisation.

Random amplified polymorphic DNA (RAPD) markers distinguished among asparagus cultivars, and revealed differences within seed-raised commercial cultivars. The RAPD

technique also detected changes in genomic DNA structure induced during culture of embryogenic cells. No change in genomic structure of plantlets regenerated from somatic embryos was detected.

Cytological analysis, using chromosome counts and DNA content analysis, were used to determine the genetic stability of embryogenic calli, suspension cells, and plantlets regenerated through somatic embryogenesis. The basic chromosome number of 20 (2n = 20) remained unchanged for all samples. The DNA content of explants and plantlets was similar, indicating that plantlets were diploid. The experiment was unable to detect somaclonal variation, revealed by altered ploidy level indicating that cytological analysis is not as sensitive as RAPD analysis for detecting somaclonal variation.

Extracellular protein profiles generated for embryogenic cells grown in suspension culture were influenced by PGR concentration and length of time in culture, and were therefore not suitable for monitoring somaclonal variation.

Overall, individual cultivars produced between 6 to 8 different calli types for all PGR treatments. Plant genotype and PGR treatment influenced the phenotype of calli developed for each cultivar. The results indicate that, for the six asparagus cultivars investigated in this study, nodular calli or nodular mucilaginous calli have more embryogenic potential than other calli types. These calli were also noted to produce embryogenic cells in suspension, and could, therefore, be used to successfully inoculate liquid cultures either for small or large-scale production of asparagus somatic embryos.

Keywords

Asparagus officinalis L., *in vitro*, plant growth regulator, somatic embryos, embryogenic calli, maturation, regeneration, plantlet acclimatisation, random amplified polymorphic DNA (RAPD), chromosome count, DNA content, extracellular protein

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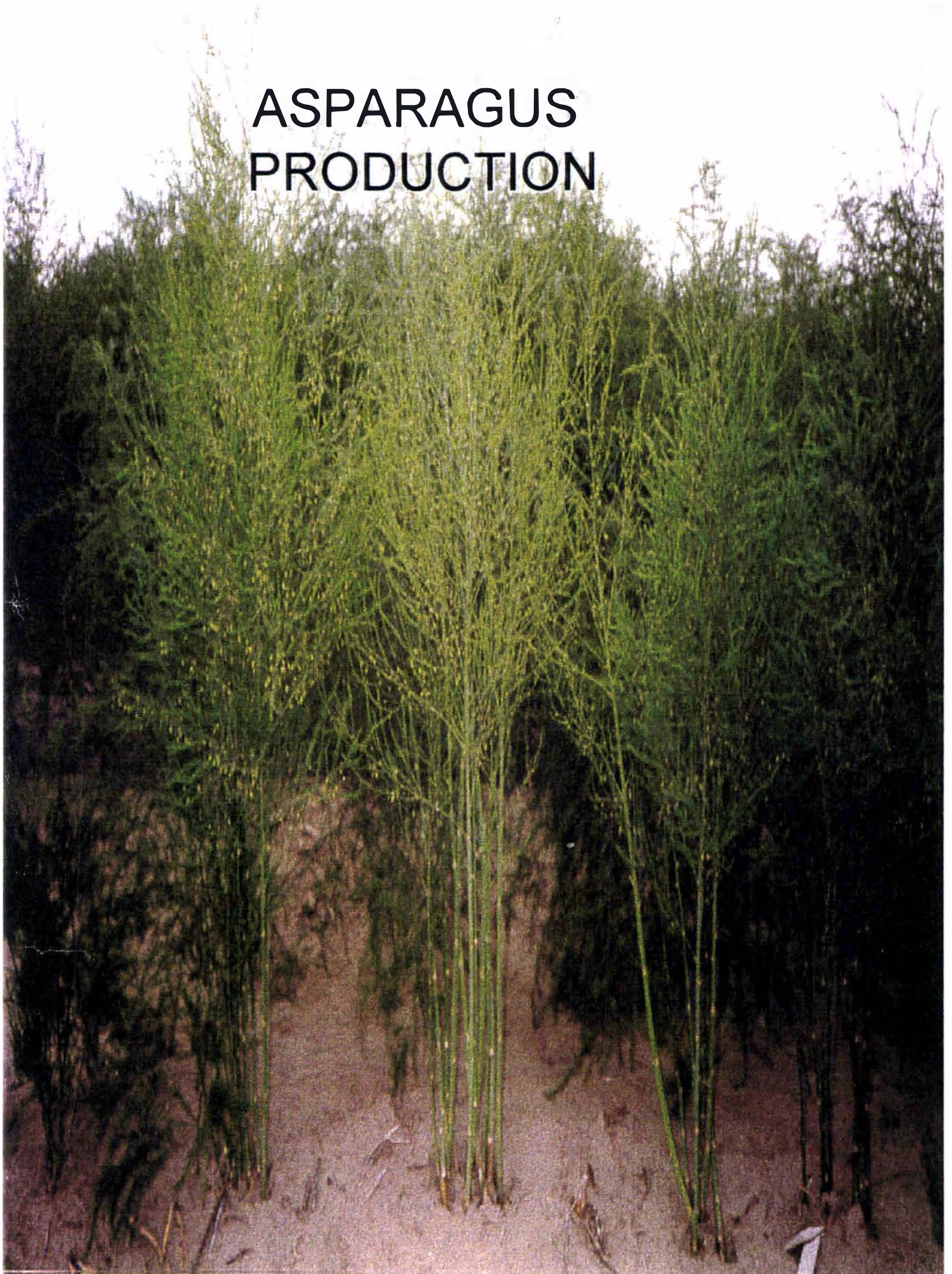
List of Abbreviations

µg	microgram (s)
µl	microlitre (s)
µm	micrometer (s)
µM	micromolar (s)
µmol	micromole (s)
2,4-D	2,4-dichlorophenoxyacetic acid
2C	nuclear DNA content of unreplicated diploid chromosome complement
ABA	Abscisic acid
AFLP	Amplified fragment length polymorphism
ASP	Asparagus cultivar Aspiring
BA (BAP)	Benzylamino purine
CFLP	Cleavase fragment length polymorphism
Chl-	Chlorophyll deficient embryos
Chl+	Globular embryos containing chlorophyll
CI	Callus induction
CRD	Complete random design
DNA	Deoxyribonucleic acid
EI	Embryo induction
g	grams
GA_3	Gibberellic acid
hr	hour (s)
IAA	3-indole acetic acid
IEDC	Induced embryogenic determined cell
kD	kilodalton
kg	kilogram (s)
Kn	Kinetin
KP	Asparagus cultivar Karapiro
l or L	litres
LEA	Late embryogenesis protein
mg	milligram (s)
min	minute (s)
mm	millimetre (s)
MS	Murashige and Skoog
MW	molecular weight
NAA	α-Napthaleneacetic acid
°C	degrees Celsius
PC	Asparagus cultivar Pacifica
PCR	Polymerase chain reaction
PEDCs	Pre-embryogenic determined cell
PEG	Polyethylene glycol
PEMs	Proembryogenic masses
PGR	Plant growth regulator (s)
PI	Propidium iodide
Pur	Globular embryos with purple pigment
RAPD	Random amplified polymorphic DNA

RFLP	Restriction fragment length polymorphism
Rg	Regeneration
SEM	Standard error of mean
SN	Asparagus cultivar Syn4
SSCP	Single stranded conformation polymorphism
TU	Asparagus cultivar Turoa
UC	Asparagus cultivar UC157

SECTION 1

ASPARAGUS PRODUCTION



CHAPTER 1
GENERAL INTRODUCTION

1.1 ASPARAGUS PRODUCTION

1.1.1 Asparagus distribution

Asparagus is the generic name of approximately 300 species comprising the Asparagus Genus in the Liliaceae family (Douglas 1990). Some species are of horticultural importance, for example, *Asparagus plumosus* and *Asparagus meyerii* which are ornamental plants, while *Asparagus officinalis* L. is the economically important vegetable asparagus.

This research project is concerned with the micropropagation of *Asparagus officinalis* L. a native to south-central Europe, Western and Central Asia, and North Africa (Fehér 1992). While the above ground plant part is edible, the underground rhizomes are occasionally used in herbal medicines, and can be traced to civilisations as far back as 2200 BC (Douglas 1990). *Asparagus officinalis* L. is commonly grown in temperate regions where it undergoes a temperature controlled dormancy. However, recently, production has expanded to tropical regions where growth remains active year round (Desjardins 1992).

1.1.2 International asparagus production

Asparagus is produced for the fresh vegetable market and for processing (frozen, dehydrated or canned). As shown in Table 1.1, the largest growers producing >10 000 hectares (ha), are the USA; the European Economic Community (EEC) including France, Italy, Spain, Germany and the UK (Desborough 1979; Hobson 1982; Desjardins 1992); and China, Japan, and Mexico (Nigh 1997).

Asparagus production in New Zealand has increased over the past 2 decades from approximately 600 ha under cultivation in 1980 to about 3500 ha in 1990 with a total

marketable yield between 8000 and 10 000 tonnes (Nichols 1990). Asparagus yield in New Zealand ranges from 2.4 to 4.7 tonnes/ha depending on the production site (McCormick & Franklin 1990). Data for 1989 show that approximately 64% of asparagus produced in New Zealand was processed, 22% exported fresh and 14% consumed fresh locally (Nichols 1989).

Table 1.1 International asparagus production in 1990 (Desjardins 1992) and 1997 (Nigh 1997).

Country	Production area (ha) 1990	Production area (ha) 1997
China	-	50 000
USA	45 870	39 500
Spain	22 000	19 000
France	20 000	12 800
Taiwan	10 000	-
Japan	9 970	11 000
Italy	6 000	6 000
Germany	5 200	9 100
Chile	4 300	5 470
Australia	3 510	3 000
Mexico	3 100	10 830
New Zealand	3 000	3 000
The Netherlands	2 840	2 300
Argentina	2 840	1 645
Canada	2 000	1 085
Others	25 840	41 216
Total production	169 570	215 946

1.2 ASPARAGUS BREEDING AND PROPAGATION

1.2.1 Asparagus genome

Asparagus is a dioecious perennial with chromosome number 2n=2x=20 and a haploid genome size of 1.8 x 10^6 kbp (Bracale *et al.* 1991). Female plants (pistillate plants) are homogametic (xx) while male plants are either heterogametic (xy) or homogametic (yy) (Bracale *et al.* 1990; Desjardins 1992). Occasionally, a functional pistil may form on a staminate plant, producing andromonoecious plants with hermaphrodite flowers. Upon fertilisation these flowers produce only male genotypes, xy males and yy supermales (Bracale *et al.* 1991). Staminate plants are commercially desirable because of their greater yield and vigour. Low yield and vigour in pistillate plants may be partially

attributed to the partitioning of energy reserves to produce flowers and fruit (Lazarte & Garrison 1980).

As populations of *Asparagus officinalis* L. seedlings are highly heterozygous, pure inbred homozygous lines are difficult to develop. Therefore, individual seeds harvested from the same plant normally have different genotypes, a variability that can adversely affect the yield of asparagus production fields because the productivity of individual plants may differ markedly. However, techniques such as *in vitro* anther culture can be used to develop homozygous lines of select supermales for commercial use (Falavigna *et al.* 1990). Corriols *et al.* (1990) report the first commercial release of a homozygous all-male F_1 asparagus hybrid developed in their breeding program, using homozygous diploids parents.

1.2.2 Conventional asparagus propagation

Traditionally, asparagus fields were established either from dormant one year old crowns, by direct seeding, or by 10-12 week old seedling transplants (Alder *et al.* 1985). Asparagus crowns used as planting stock for establishing commercial fields are obtained by sowing seeds into nursery stock beds in the spring. Dormant crowns are dug in autumn to early winter and planted out the following winter or early spring (Nichols 1989). Ideally, crowns should be of uniform size, 25-30 mm across the bud cluster, with firm white fleshy roots 200 mm long (McCormick & Franklin 1990). Direct seeding *in situ* is used to establish high density stands, but is no longer a common practice because of the high cost of hybrid seed and difficulties involved in weed control in young seedlings (Nichols 1989). Seedlings with active fern growth are transplanted from Speedling® trays grown in semi-controlled greenhouse conditions for 10-12 weeks (Nichols 1989; McCormick & Franklin 1990).

Seedling transplants have a number of advantages over crowns and direct seeding for they extend spring planting; can be planted mechanically; are grown in sterile media; and, if grown to specification, have unbroken root systems which reduce infection from soil borne fungal diseases. Because seedlings have live fern growth and are smaller than

crowns, they require more care when transplanted. Normally, asparagus beds established by seedling transplants require two full growing seasons before harvest. In contrast, crowns require only one full growing season (McCormick & Franklin 1990). Seedling transplants have the additional problem that their fern growth can be damaged by machinery during transplanting (Alder *et al.* 1985).

1.2.3 Non-conventional asparagus propagation

To avoid yield variation expected from the use of heterozygous seedlots in asparagus fields, asparagus propagators and growers in New Zealand and overseas have used micropropagation techniques to multiply individual high producing plants (McCormick & Franklin 1990; Desjardins 1992). Initially, interest in asparagus tissue culture focused primarily on supplying breeding programmes with large numbers of identical clones for hybrid breeding production (Lazarte & Garrison 1980; Desjardins 1992). However, today emphasis is placed on utilising micropropagation techniques to mass produce high yielding asparagus cultivars for field establishment.

A number of techniques are used in plant micropropagation and each can be utilised to produce asparagus clones, including, shoot tip, meristem and adventitious shoot cultures, protoplast, haploid cell culture and somatic embryogenesis. The following sections will discuss each procedure and their relative merits and disadvantages.

1.2.3.1 Asparagus shoot tip culture

Micropropagation using shoot tip culture promotes the growth and multiplication of terminal and axillary shoots. Microcuttings are usually about 2 mm in length, but can be 10 mm to 20 mm or longer (Hartmann *et al.* 1990). Shoot tip growth is normally induced by relatively low levels of cytokinins (0.1 to 1 mg/L) in the growth medium (Yang & Clore 1974; Conner & Falloon 1993). Asparagus shoot tip culture was first reported by Loo (1945) who demonstrates that it is possible to grow asparagus stem tips continuously on a basal medium supplemented with sucrose, and inorganic and organic constituents. However, Loo was unable to stimulate root growth on regenerated plantlets. Morel & Wetmore (1951) present evidence showing that it is possible to

initiate, and develop, root growth on *in vitro*-cultured asparagus and other monocotyledons. Since these initial reports, asparagus shoot tip culture has been studied extensively (Groter 1965; Yang & Clore 1973, 1974).

1.2.3.2 Asparagus meristem tip culture

This method of *in vitro* propagation uses the meristematic dome plus a few of the surrounding leaf primordia from excised shoots (Hartmann *et al.* 1990). Meristem tip culture, unlike shoot tip culture, is frequently used to eliminate viruses from plants. Asparagus meristem culture was utilised by Murashige *et al.* (1972) to develop nutrient components that promote the rapid formation of new spears and roots in shoot apices excised from buds and lateral branches. Further, Hasegawa *et al.* (1973) show that adventitious roots developed from callus tissue formed at the base of explants, while any spears that develop originate from axillary buds. However, meristem tip culture can be labour intensive and costly, mainly because new apices need to be continuously collected from spears growing in fields or greenhouses (Desjardins 1992).

1.2.3.3 Asparagus adventitious shoot culture

Adventitious shoot culture, unlike meristem tip or shoot tip cultures, depends on adventitious shoots initiated directly on explants that are normally obtained from pieces of leaves, shoot tip, cotyledons, hypocotyls, bulb scales or pieces of immature inflorescence (Hartmann *et al.* 1990). In contrast to shoot tip culture, induction of adventitious shoot culture usually requires a higher cytokinin concentration in the growth medium. Takatori *et al.* (1968) demonstrate both the possibility of producing calli from asparagus spear slices, and the ability to induce plantlet formation directly from these calli. However, adventitious shoot formation from callus cultures is a common occurrence and is used either for fundamental research or for breeding programmes (George & Sherrington 1984).

1.2.3.4 Asparagus protoplast culture

As protoplasts culture uses isolated single somatic cells without their cell wall (Cocking 1972), plant regeneration through protoplast culture has many advantages for breeding programmes, primarily for gene transformation and somatic hybridisation (Kunitake & Mii 1990). Although regeneration from protoplasts have been reported for some economically important crops (Mórocz *et al.* 1990; Hartmann *et al.* 1990; Tahara *et al.* 1994), widespread applicability has been limited by the lack of efficient protocol development. Another limiting factor is the high percentage of somaclonal variation recorded in plants regenerated from protoplasts (George & Sherrington 1984). Initially, the successful culture of asparagus protoplasts was made by Bui-Dang-Ha *et al.* (1975). Since these early attempts, many researchers have developed various protocols for isolation, culture and transformation of asparagus protoplasts (Delbreil *et al.* 1993; Mukhopadhyay & Desjardins 1994c; Guangyu *et al.* 1996; May & Sink 1996).

1.2.3.5 Haploid cell culture of asparagus

Pollen, anther, and unfertilised ovule culture use haploid gametophytic tissue to produce vegetative tissue (George & Sherrington 1984). Since the culture of haploid tissue was first reported by Tulecke (1953) it has become an important component of the breeding program of several plant species (Beasley *et al.* 1974; Falavigna *et al.* 1990). Homozygous plants are produced following chromosome doubling of haploid tissue (Sunderland & Dunwell 1977). For the production of asparagus, anther and pollen culture (androgenesis) is more common than ovule culture, mainly due to the interest in producing all-male hybrids for breeding programmes (Falavigna *et al.* 1990, 1996). An additional advantage to using androgenesis to produce asparagus clones is that after chromosome doubling (which can occur naturally in the callus), homozygous plants are produced. This is of particular importance as asparagus is a cross-pollinated plant and F_1 plants are highly heterogeneous (Ellison 1986).

1.2.3.6 Asparagus somatic embryogenesis

Plant regeneration by somatic embryogenesis depends on the development of fully functional 'seed-like' embryos from somatic cells during *in vitro* culture (Wann 1988; Hartmann *et al.* 1990; Thanh 1992). Unlike organogenesis, which develops unipolar shoot or root primordia, embryogenesis produces bipolar structures with both a shoot and root axis (Thorpe 1990). Wilmar and Hellendoorn (1968) were the first to report the production of somatic embryos and plant regeneration of *Asparagus officinalis* L. Since this investigation, much research has been undertaken to improve the efficiency of embryo development and plant regeneration (Levi & Sink 1990; Saito *et al.* 1991; Wolyn & Feng 1993).

Production of asparagus clones by somatic embryogenesis has many advantages over the other micropropagation techniques discussed above, including:

- the potential for simultaneous development of shoot and root primordia, thereby eliminating the need to transfer developing embryos onto different media for shoot and root development;
- the production system can easily be adapted to suspension culture with the potential for large scale bioreactor production and development of a fully automated process;
- the labour intensive step of subculturing large numbers of plantlets is eliminated;
- the system has the potential to be adapted to synthetic seed technology; and
- the occurrence of genetic variability is reported to be very low using an embryogenic pathway to regenerate plantlets (Thorpe 1990; Reuther 1996).

As these advantages have the potential to make somatic embryogenesis a superior micropropagation technique for producing asparagus clones, this research project investigates the potential of using somatic embryogenesis for the commercial production of selected asparagus cultivars.

1.3 CLONAL PROPAGATION - ASPARAGUS PRODUCTION IN NEW ZEALAND

Recent advances in asparagus micropropagation has improved the possibility of using this technique to establish large commercial fields of high yielding, uniform asparagus plant stands (McCormick & Franklin 1990; Abernethy & Conner 1992). Utilisation of asparagus clones offers many advantages including: the potential for earlier harvest after field establishment; improved uniformity in field establishment, as well as improvement in spear colour, shape, length and diameter at harvest; and, increased yields (Wilson 1991; Abernethy & Conner 1992).

In 1987 the New Zealand Asparagus Council, in a joint research venture with growers and the New Zealand Crop and Food Research Ltd. Lincoln, Christchurch (formerly Crop Research Division, D.S.I.R), initiated a research project to identify and select superior performing asparagus plants from growers' fields. This project has identified three clones reported to produce yields up to 400% more than the most popular cultivar 'Jersey Giant' (Reddish 1995). Spears produced by these clones were found to meet the high, export quality standards.

Commercial micropropagation of these 'superclones' was initially by meristem tip culture followed by shoot tip and or lateral bud culture for mass production (Abernethy & Conner 1992). However, because this process is labour intensive and costly, clones are relatively high priced. For example, using conventional propagation, crowns cost between NZ$ 0.10 to $0.27 (Broom 1992), seedlings cost about $0.25 and each clone between $1.40 to $2.00 (Wilson 1991). Prices for each cultivar vary depending on the ease of propagation. With expected increased yields and improved quality it was predicted that the new cultivars would allow growers to produce as much asparagus on 10 hectares as they currently produce on 40 ha using the standard cultivars (UC157 and Jersey Giant). As yields would be so much higher, growers could recoup plant costs in four years after new clone establishing fields (Wilson 1991). In reality, growers have not taken up this idea because of the high establishment costs and the uncertainty about yield increases.

However, like all technologies, there are some problems limiting the use of micropropagation, especially at the commercial level. The most important of these limitations include:

- high cost of research and development, facilities, equipment and personnel;
- the commercial adaptability of some techniques, especially for difficult-to-propagate cultivars;
- acclimatisation of plantlets transferred from the controlled tissue cultured environment to the greenhouse and subsequently to the field;
- species and cultivar specific responses that makes it difficult to develop generic procedures for crops; therefore for many plant species, production protocols must to be developed or modified for each cultivar;
- somaclonal variation can result in variants being produced, while this may be useful from a breeding point of view, it can be detrimental if the sole objective is to commercially produce clones of a specific cultivar; and
- the high risk of contamination which can cause a large number of plants to be lost in a short time (MacDonald 1986; Hartmann *et al.* 1990; Pierik 1991).

1.4 RESEARCH PROBLEM, AIM AND OBJECTIVES

1.4.1 Research problem

While asparagus clones continue to be produced through *in vitro* culture of shoot tip or lateral buds, further research is required to make commercial propagation more cost effective. Regeneration of *Asparagus officinalis* L. clones via *in vitro* somatic embryogenesis has the potential to produce asparagus plants at a relatively low cost. However, this technique has never been developed for commercial use as there are some problems associated with the process including embryo conversion, *ex vitro* acclimatisation of regenerated plantlets, and somaclonal variation. This research focused on addressing these problems.

1.4.2 Research aim

This research project investigated the potential for producing clones for six asparagus genotypes by somatic embryogenesis. The aim was to study the factors influencing the production of somatic embryos and the occurrence of somaclonal variation of embryogenic calli and regenerated plantlets.

1.4.3 Research objectives

There were two main objectives:
The first objective involved the development of protocols for the initiation, development and conversion of asparagus somatic embryos, as well as acclimatisation of plantlets regenerated from somatic embryos. This objective was achieved through optimisation of media for embryogenic calli initiation (experiment 1), embryo induction, maturation and embryo conversion to plantlets (experiment 2). In addition, several different acclimatisation techniques were examined to improve survival of plantlets transferred to the greenhouse (experiment 3).

The second objective was to determine the genetic stability of the somatic embryogenic process for the commercial production of specific asparagus clones. This was achieved by analysing explants, calli and plantlets using the following techniques:

- the polymerase chain reaction (PCR)-based technique, random amplified polymorphic DNA (RAPD) analysis, was used to detect changes in DNA profiles, of samples, that may occur during somatic embryogenesis (experiments 4 & 5);
- cytological analysis was used to monitor changes in chromosome number and DNA content of cell lines developed during long-term culture of embryogenic cells (experiment 6);
- the potential of SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) to reveal changes in the extracellular protein and glycoprotein profile of each cell line was investigated (experiment 7).

SOMATIC EMBRYOGENESIS

Asparagus somatic embryogenesis: 2. Somatic embryo development, maturation and regeneration

Abstract

Several somatic embryo induction, maturation and regeneration protocols were investigated for their effectiveness to promote embryo development from embryogenic calli developed from three different asparagus genotypes. The three cultivars used in this experiment were Aspiring (ASP), Karapiro (KP) and Pacifica (PC). The following sequence was found to be most effective in producing complete plantlets from embryogenic calli: callus induction (CI) on Murashige and Skoog (MS) media containing 3% sucrose, 1% agar and either of 1.0, 3.0 and 10 μ 2,4-D, followed by transfer onto liquid embryo induction media (EI) containing MS + 6% sucrose and finally regeneration on regeneration media (Rg4) containing MS + 0.2 g/l glutamine + 3% sucrose + 1% agar. Globular embryos were produced by all cultivars; however, only globular embryos developed from cultivar PC converted to plantlets. Treatment of 'PC' globular embryos at -15^{0}C (due to equipment failure) produced the highest percent converted plantlets (34 and 26% for 6-month-old embryogenic calli and 1 year-old embryogenic suspension cells respectively). Precocious germination, rhizogenesis, recurrent embryogenesis and organogenesis were common problems encountered. Further studies are required to understand the molecular and biochemical events involved in triggering and switching off embryogenic gene expression.

Keywords

Asparagus officinalis, abscisic acid (ABA), osmotic potential, polyethylene glycol (PEG), embryo conversion, recurrent embryogenesis, rhizogenesis, organogenesis, precocious germination

CHAPTER 2
SOMATIC EMBRYOGENESIS

2.1 INTRODUCTION

Somatic embryogenesis is analogous to zygotic embryogenesis, except that the embryo is formed from vegetative cells (single cells or a small group of cells) and is not the product of gametic fusion (Tautorus *et al.* 1991). This phenomenon occurs naturally and is referred to as adventitious embryony. Somatic embryogenesis can also occur *in vitro* under specific conditions (Merkle *et al.* 1995). The following sections will first, briefly describe the natural occurrence of somatic embryogenesis. As this research project focuses on production of asparagus clones by somatic embryogenesis, attention will then be focused on *in vitro* induced somatic embryogenesis and the factors affecting this process.

2.1.1 Naturally occurring somatic embryogenesis

In higher plants, zygotic embryos are formed by sexual reproduction involving the fusion of one sperm cell and an egg cell (Dudits *et al.* 1995). In some plants, e.g. the tetraploid Eastern gamagrass (*Tripsacum dactyloids* var *dactyloids* (L.) L.), viable somatic embryos may be formed asexually (Kindiger & Dewald 1996). This form of asexual reproduction is referred to as apomixis and results in the production of plant populations genotypically identical to the parent stock. Apomixis is divided into two main categories: vivipary, which refers to vegetative reproduction involving the formation of new plants such as bulbils; and agamospermy (Sharma & Thorpe 1995). As *in vivo* somatic embryos are formed through the mechanism of agamospermy, only this category will be further discussed. Agamospermy leads to polyembryony where one or more asexual embryo can exist along with the zygotic embryo, and includes adventitious embryony and gametophytic apomixis. In adventitious embryony the embryo is formed directly from somatic cells in the ovule without the formation of embryo sac and egg cell. Gametophytic apomixis involves the formation of the female gametophyte from an unreduced embryo-sac initial (diplospermy) or from somatic cells in the nucellus or

chalaza (apospermy). Both diplospermy and apospermy result in parthenogenesis. Apomictic embryos develop within the seed and may be phenotypically indistinguishable from zygotic embryos (Krishnaraj & Vasil 1995).

Natural asexual embryogenesis occurs in both angiosperms and gymnosperms and involves reproductive tissues including the nucellus, synergids, antipodals, endosperm, zygote and suspensor (reviewed in Sharma & Thorpe 1995).

2.1.2 *In vitro* somatic embryogenesis

Adventitious embryony, also known as somatic embryogenesis, depends on the formation of functional embryos from somatic cells or from cultured cells and tissues. *In vitro* somatic embryogenesis was first described in the 1950s using carrot callus and suspension cultures (Levine 1950; Steward *et al.* 1958a and 1958b). These early observations reveal that complete plantlets could be regenerated from carrot callus cultures. However, while the conditions required for the development of plantlets varied among researchers, it was noted that plantlets, or some kind of organised cellular development, could be achieved by first growing callus on a medium supplemented with plant growth factors. On transfer to a medium lacking these growth factor(s), embryo regeneration could be observed (Levine 1950; Steward *et al.* 1958a and 1958b). While making a detailed description of the development of free cells in carrot suspension cultures, Steward *et al.* (1958a) allude to, but did not specifically identify, the ontogeny of embryogenic cells. It was not until later that the "small isodiametric cells, which tended to aggregate into multi-cellular masses", described by Steward *et al.* (1958a), were referred to as being embryogenic. In a further study Steward *et al.* (1958b) observed the development of roots and shoots from tissue aggregates derived from free cells in suspension.

Building on the foundation of these pioneer works, researchers have developed somatic embryogenic systems for a wide range of plant species, including gymnosperm species and monocotyledonous and dicotyledonous angiosperms (Maheswaran & Williams 1984; Hakman *et al.* 1985; Hakman & von Arnold, 1985; Lupotto 1986; Gupta & Durzan 1987; Vasil 1988; Attree *et al.* 1990a; Conger *et al.* 1989; Tautorus *et al.* 1991; Dhanalakshimi

& Lakshmanan 1992; Levi & Sink 1992). *In vitro* somatic embryogenesis has also been the focus of many reviews (Gupta *et al.* 1993; Lindsey & Topping 1993; Dudits *et al.* 1995; Halperin 1995).

2.2 SOMATIC EMBRYOGENIC PROCESS

In vitro somatic embryos can be produced from cells initiated from a number of explant sources including vegetative cells of mature plants, roots, diploid cells, mature or immature zygotic embryos, epidermal cells of the hypocotyl and tissues of the nucellus and synergids (Smith & Krikorian 1989; Klimaszewska 1989; Becwar *et al.* 1991; Dhanalakshmi & Lakshamanan 1992).

The origin and classification of embryogenic cells are determined by the type of explant used during the initiation phase. Embryogenic cells derived from a non-embryogenic cell source are often referred to as 'induced embryogenic determined cells' (IEDCs). Alternatively, embryogenic cells originating from cells obtained from plant embryos already expressing embryogenic genes, are referred to as 'pre-embryogenic determined cells' (PEDCs). The origin of embryogenic cells also distinguishes direct and indirect embryogenesis. Direct embryogenesis normally involve the stimulation of cell division in PEDCs. In this case, somatic embryos can be produced directly on the explant without an intervening callus phase (Williams & Maheswaran 1986; Pretova & Williams 1986; Tetu *et al.* 1990; Denchev *et al.* 1992). Direct embryogenesis has also been recorded on explant material not expressing PEDCs (Hilbert *et al.* 1992). Induced embryogenic determined cells require exogenous growth regulators, or other 'inducing' factors, to initiate mitotic cell divisions that can lead to the production of pro-embryogenic cell masses, and eventually somatic embryos. This process involves an intervening callus phase and is commonly referred to as indirect embryogenesis (Merkle *et al.* 1995).

During the early stages of embryogenesis, the developmental pattern of somatic and zygotic embryos are similar (Becwar *et al.* 1987). For the purpose of this review the *in vitro* embryogenic process will be discussed under the following sections:

- callus induction,

- selection and development of an embryogenic cell population, and
- embryo maturation and conversion.

Factors affecting these stages will also be addressed.

2.2.1 Induction of embryogenic calli

The onset of somatic embryogenesis requires the genetic reprogramming of existing cells within the explant so that they become embryogenic. Reprogramming involves the termination and replacement of developmental gene expression with embryogenic gene expression (Merkle *et al.* 1995). At the cellular level, this change in gene expression results in de-differentiation of pre-programmed organised cell development to a meristematic state. These meristematic cells either continue their pre-programmed development or they develop into embryoids (Jones 1974). This switch to embryogenic gene expression within somatic cells is believed to be an adaptive response to severe stress conditions (Dudits *et al.* 1995). Experimental evidence indicates that high concentrations of an auxin, such as 2,4-dichlorophenoxyacetic acid (2,4-D), in the culture medium acts as a stress inducing factor to initiate somatic embryogenesis (Dudits *et al.* 1991). *In vivo* studies investigating the response of whole plants to exogenous auxin application have shown that the presence of auxin induces changes in gene expression and protein synthesis within minutes of its application (reviewed in Brummell & Hall 1987). These changes are also observed to occur during the early stages of somatic embryogenesis.

Other plant growth regulators used for induction of asparagus (and other plant) embryogenic calli include napthaleneacetic acid (NAA); indole acetic acid (IAA); kinetin; and benzylamino purine (BAP) (Levine 1950; Maheswaran & Williams 1984; Schuller *et al.* 1989; Nørgaard & Krogstrup 1991; Mukhopadhyay & Desjardins 1994a and 1994b). Additional, essential media components include basic micro- and macro-nutrients plus vitamins (Murashige & Skoog 1962; Murashige *et al.* 1972; Schenk & Hildebrant 1972) and a carbohydrate source such as sucrose or glucose (Loh *et al.* 1983; Levi & Sink 1992); potassium and ammonium ions (Tazawa & Reinert 1969; Brown *et al.* 1976); nitrogen (Wetherell & Dougall 1976); and amino acids (Trigiano & Conger 1987).

Several non-hormonal stress inducing factors have trigger the reprogramming of embryogenic competent cells to become embryogenic, including wounding the explant (Smith & Krikorian 1989 and 1990; Dudits *et al.* 1995); heat stress (Zimmerman *et al.* 1989; Hilbert *et al.* 1992); electrical stimulation in protoplast culture (Dijak & Simmonds 1988); cold storage (Hakman & von Arnold 1985); metal ions such as cadmium and some ethylene inhibitors (Roustan *et al.* 1989); high salt concentrations; and high osmotic potential (Binh & Heszky 1990; reviewed in Merkle *et al.* 1995).

Several factors affect the successful establishment and maintenance of embryogenic cultures *in vitro*, including the developmental stage, age and physiological condition of the explant (Vasil 1988; Attree *et al.* 1990a; Karunaratne *et al.* 1991). Explants can consist of cells which may range from a PEDCs (e.g. zygotic embryos), to non-embryogenic cells (e.g. in an established seedling) (Merkle *et al.* 1995). Therefore, the nature of the explant influences whether embryogenesis occurs directly or indirectly. Callus phenotype also influences the efficiency of embryogenesis. Of the two types of calli derived from Norway spruce immature zygotic embryos, the white translucent embryogenic callus was observed to have a higher embryogenic capacity than green callus (Becwar *et al.* 1987). Yellow mucilaginous calli were more effective at producing asparagus somatic embryos than yellow friable callus (Levi & Sink 1991b). Plant genotype also influences the efficiency of somatic embryogenesis (Vasil 1988; Krishnaraj & Vasil 1995). However, factors such as developmental stage and physiological state of the explant, explant growth environment and nutrient regime have been noted to overcome genotypic effects.

2.2.2 Somatic embryo development

Embryogenic cells, once formed, are capable of synthesising auxin in the form of indole acetic acid (IAA); therefore, their dependence on exogenous auxin supplies is reduced (Zimmerman 1993). While embryo development from single cells or PEMs through to early globular stage can occur in the presence of auxin, further development and maturation of embryos, requires removal, or a significant reduction, of auxin (Borkird *et al.* 1986; Zaghmout & Torello 1988).

It is thought that 2,4-dichlorophenoxyacetic acid triggers an IAA biosynthetic pathway resulting in the production of endogenous tryptophan-derived IAA. This is only active during callus proliferation for it appears to be 'switched off' during somatic embryo formation. These results suggest that high levels of endogenously synthesised IAA favour the maintenance and proliferation of unorganised callus growth and prevent the development of somatic embryos. Early events leading to the induction of carrot somatic embryogenesis occur in the presence of high levels of IAA (Michalczuk *et al.* 1992a). Therefore, it is plausible that low levels of endogenous IAA *not* exogenous 2,4-D, may be essential for the development of somatic embryos beyond the globular stage. Michalczuk *et al.* (1992b) suggest that low levels of endogenous auxin may be required by globular stage embryos to establish and maintain polarised growth. However, the precise mechanism by which exogenous 2,4-D and endogenously synthesised IAA influences somatic embryo development has yet to be clarified.

Because embryogenic cell cultures may proliferate for long periods of time (Fujimura & Komamaine 1980; Dhanalakshimi & Lakshmanan 1992), this phenomenon is commonly referred to as repetitive or recurrent embryogenesis (Lupotto 1986; Merkle 1995). A callus mass or suspension culture contains a heterogeneous population of embryogenic and non-embryogenic cells of various sizes (Hakman & Fowke 1987; Komamine & Kawahara 1993; Halperin 1995).

Once formed, somatic embryos go through a series of developmental and morphological stages similar to those of zygotic embryos. Monocotyledonous embryos, e.g. asparagus, normally develop through globular, coleoptilar and scutellar stages, while embryos from dicotyledons and conifers develop through globular, heart, torpedo and cotyledonary stages (Merkle *et al.* 1995; Tautorus et al. 1995).

Komamine and Kawahara (1993) outlined four phases of somatic embryogenesis for carrot suspension cultures. Investigation of embryo development revealed that in the first stage (phase 0), competent cells divided asymmetrically in the presence of auxin, to produce embryogenic cell clusters. On transfer to auxin-free medium, these cell clusters proliferated slowly to produce pro-globular embryos of phase 1. Rapid cell division in

these pro-embryos resulted in the formation of globular embryos in phase 2. The next stage (phase 3) involved further embryo development from heart shaped embryo to torpedo shaped embryo and finally embryo regeneration to form complete plantlets. The stages of somatic embryo development of carrot cell cultures have also been examined by geometric analysis (Schiavone & Cooke 1985) confirming that carrot somatic embryos normally progressed through the sequence of stages outlined by other investigators (Fujimura & Komamine 1980). However, Schiavone and Cooke (1985) demonstrate that an intermediate class of embryos occur between globular and heart stage embryos. Because of their shape, these embryos are referred to as oblong embryos.

Several factors influencing the multiplication and development of somatic embryos include plant species and genotype, concentration of exogenous and endogenous plant growth regulators, other medium supplements and the culture environment (Borkird *et al.* 1986; Smith & Krikorian 1990; Michalczuk et al. 1992a and 1992b).

2.2.3 Embryo maturation

Development of bipolar somatic embryos is followed by a period of maturation and developmental arrest (West & Harada 1993). This maturation stage is essential because it is at this stage proteins, carbohydrates and lipids reserves are accumulated. Failure to store these reserves normally results in the following problems: precocious germination, which produces large numbers of malformed embryos; failure of embryos to germinate; and poor *ex vitro* acclimatisation of plantlets (Merkle *et al.* 1995; Reuther 1996). At this stage, development of somatic and zygotic embryos differ. Zygotic embryo development is characterised by the production and mobilisation of storage proteins, carbohydrates, lipids and other compounds essential for efficient germination of embryos (West & Harada 1993; Misra 1994). This period of accumulation of storage reserves is generally followed by a period of desiccation and dormancy (quiescence). By contrast, somatic embryos do not accumulate sufficient of these reserves to support embryo conversion (germination and formation of complete plantlets with both roots and shoots) neither do they undergo a quiescent period (Dahmer *et al.* 1991; Zimmerman 1993). While there are few reports giving an account of storage reserves in mature asparagus somatic embryos

(Reuther 1990a and 1996), they are not comprehensive, resulting in the view that more studies are required to better understand the factors influencing asparagus somatic embryo maturation.

Precocious germination, investigated by several researchers (Attree *et al.* 1990b; Anandarajah *et al.* 1991; Dunstan *et al.* 1992; Misra *et al.* 1993), indicate abscisic acid (ABA) and high osmotic potential play an essential role in the accumulation and storage of reserves required for somatic embryo maturation and conversion (Dunstan *et al.* 1988; Jain *et al.* 1988). Additionally, abscisic acid inhibits cleavage polyembryony, promoting singulation and maturation of somatic embryos (Dunstan *et al.* 1991; Gupta *et al.* 1993). The length of exposure of embryos to ABA, and ABA concentration, are important factors influencing the percentage of healthy embryos converted to plantlets. For example, both black and white spruce somatic embryos cultured on a medium supplemented with 8-16µM ABA for 28 days yielded more matured embryos (6-8%) than embryos cultured in 24-32µM ABA for the same time (Attree *et al.* 1990b).

Abscisic acid alone does not promote the synthesis and prolonged accumulation of the right storage reserves required for embryo conversion as reports show that high osmolarity is also beneficial to asparagus somatic embryo maturation and development (Levi & Sink 1992; Mukhopadhyay & Desjardins 1994b). Attree *et al.* (1991) recorded a 3-fold increase in white spruce somatic embryo maturation when the embryos were cultured on medium with 5-7.5% polyethylene glycol 4000 (PEG-4000). Treated embryos accumulated a greater quantity of storage reserves than non-treated controls. Misra *et al.* (1993) also found that non-plasmolysing osmotic agents like PEG, in combination with ABA, improved white spruce embryo maturation. These treatments enhanced accumulation of storage reserves and embryo regeneration up to 81% compared to controls.

The type of agent used to achieve high osmolarity during culture also influences embryo maturation. Attree *et al.* (1991) found that white spruce somatic embryos matured better on medium containing 5-7.5% PEG 4000 and 3% sucrose than on medium containing sucrose at similar osmotic potential. Levi and Sink (1992) demonstrates that asparagus

somatic embryos matured on media containing a high carbohydrate concentration (~ 10%) followed by growth on a lower carbohydrate concentration (2%) generally resulted in a greater percent of embryo conversion. Mukhopadhyay & Desjardins (1994b) also note that transfer of asparagus somatic embryos onto a medium containing 10% glucose for 2 weeks, followed by growth on medium containing 2% sucrose, gave the highest number of bipolar embryos and plantlets.

The synergistic effect of ABA and high osmolarity is considered due to ABA initiating production of storage reserves in the developing embryo, while high osmotic potentials are required for prolonged accumulation of these reserves (Misra *et al.* 1993). In addition to ABA and high osmolarity, thermal stress treatment (30-33^{0}C) has enhanced embryo maturation and regeneration of desiccated *Brassica napus* L. embryos (Anandarajah *et al.* 1991). While the exact mechanism by which these treatments work is unknown, it is thought that thermal stress may induce endogenous synthesis of ABA within the embryo.

2.2.4 Embryo conversion

Successful somatic embryogenesis should culminate in the conversion of a large quantity of embryos. However, this is not always the case as residual culture treatment effects, particularly plant growth regulators, can have an adverse affect on further embryo development. In some plant species, the use of ABA to enhance embryo maturation also induces embryo dormancy (Merkle *et al.* 1995). In such cases, dormancy breaking treatments are required to facilitate embryo conversion, which may include cold treatment, use of gibberellic acid and desiccation. While high osmolarity is generally favourable to embryo development and maturation, prolonged embryo growth under such conditions may have an inhibitory effect on embryo conversion.

Pretreatment of embryogenic cultures with compounds to reduce the activity of some growth regulators or inhibitory substances secreted into the medium enhanced embryo conversion. For example, treatment of red fescue cultures with activated charcoal increased the regenerative capacity of somatic embryos derived from long-term embryogenic callus cultures (Zaghmout & Torello 1988). Activated charcoal may adsorb

residual 2,4-D, as well as other inhibitory substances secreted in the medium, thereby reducing their activity (Smith & Krikorian 1990). The non-selective adsorption of activated charcoal, may also reduce the activity of essential substances, resulting in precocious germination of embryos. Some reports cite the co-cultivation of somatic embryos with non-pathogenic bacteria or fungus to enhance embryo conversion (Kearney *et al.* 1991).

During regeneration, matured somatic embryos can either develop into a plant or undergo recurrent embryogenesis (Lupotto 1986). Normally, when recurrent embryogenesis occurs on the embryo at this stage, further shoot development ceases. Therefore, depending on the required outcome of the embryogenesis regime recurrent embryogenesis during embryo regeneration can be advantageous or disadvantageous. If the primary objective is to produce large numbers of plantlets for commercial purposes it is definitely disadvantageous. However, this is useful when proliferating embryogenic cultures over long periods.

Control of rhizogenesis, an alternative pathway to somatic embryogenesis, is a problem occurring in embryogenic culture (Levine 1950; Steward *et al.* 1958a). Jones (1974) proposes that this alternative pathway can occur in one of two ways. In the first instance, embryogenic competent cells develop to produce embryos where the root tips develop first, the shoot meristem then differentiates to form a callus mass from which shoots may later emerge. Alternatively, the root primordia may develop from buds in the cambium of PEMs and follow a developmental pattern similar to secondary roots.

2.3 MOLECULAR AND BIOCHEMICAL EVENTS OCCURRING DURING SOMATIC EMBRYOGENESIS

In the past, attempts to investigate somatic as well as zygotic embryogenesis have focused on morphological and physiological changes occurring at the cellular and intact embryo level. Recent attention has also focused on molecular and biochemical changes occurring during embryogenesis. Molecular and biochemical studies give some insight into the regulatory mechanisms involved in the induction of embryogenic cells from competent

somatic cells and the maintenance of embryogenic gene expression over prolonged periods.

The progress of *in vitro* somatic embryo development can be followed by examining changes occurring at the molecular level and also by investigating patterns in the accumulation of embryo specific lipids, proteins, carbohydrates and other compounds. Both biochemical and molecular events occurring during somatic embryogenesis have been recently reviewed (Dudits *et al.* 1991; Zimmerman 1993; Merkle *et al.* 1995).

2.3.1 Biochemical events

The most abundant food reserves synthesised and accumulated during the maturation of zygotic embryos are lipids, proteins and carbohydrates (Dahmer *et al.* 1991; Tautorus *et al.* 1991; Domon *et al.* 1994). Storage reserves of mature somatic embryos are markedly lower than in mature quiescent zygotic embryos.

2.3.1.1 Storage lipids

Somatic embryos accumulate storage lipids (triglycerides) and their corresponding fatty acids. A comparative investigation by Dahmer *et al.* (1991) noted that the concentration of lipids found in soybean somatic embryos ranged from 2.4 to 4.3 g/kg dry weight while those of zygotic embryos were approximately 185 g/kg dry weight. The fatty acids composition between somatic and mature seed embryos were also different but somatic embryos contained higher levels of palmitic and linoleic acids than zygotic embryos. The maturation protocol used during embryogenesis can influence the composition of lipids and fatty acids in somatic embryos. Therefore, differences in triglyceride levels and composition between zygotic and somatic embryos may be a reflection of the medium used. Embryos developed on PGR-free medium contained lipids more similar to those of zygotic embryos.

2.3.1.2 Intra-cellular storage proteins

In zygotic embryos, three major groups of proteins have been identified playing a significant role in embryo maturation and conversion. These protein groups can be classified as enzymes, structural proteins and storage proteins (Lindsey & Topping 1993) and are referred to as late-embryogenesis-abundant (LEA) proteins (Galau *et al.* 1986). The majority of LEA proteins identified in zygotic embryos are hydrophilic, with repeated amino acid sequences, and are primarily expressed during late zygotic embryogenesis (Hatzopoulos *et al.* 1990; Zimmerman 1993). Storage proteins accumulated by somatic embryos have similar functional and biochemical characteristics to those produced in zygotic embryos. The primary difference is that mature somatic embryos accumulate substantially lower quantities of these proteins (Shoemaker *et al.* 1987; Misra *et al.* 1993). The storage protein content of mature cotton somatic embryos varies with the stage of embryo development (Shoemaker *et al.* 1987), globular, heart, tulip-stage and mature embryos contained approximately 0.36%, 2.4%, 3.0% and 1.3% mature storage proteins, respectively.

Another difference between zygotic and somatic embryo development is the lack of involvement of maternal tissue in somatic embryogenesis. Therefore, it is interesting to note that similarities between protein synthesis and accumulation in both types of embryogenesis indicate that the regulation of some genes required for embryo development is possible in the absence of maternal tissue (Lindsey & Topping 1993). These LEA proteins may be associated with protecting the embryo from desiccation. In carrot and cotton somatic embryos, LEA proteins were expressed at an earlier stage than in their zygotic counterparts (Shoemaker *et al.* 1987). Studies on the timing of expression and developmental pattern of LEA proteins show they are ABA-inducible (Galau *et al.* 1986). Several embryogenesis-related proteins have been isolated from carrot embryogenic cultures including: the DC8 protein (Hatzopoulos *et al.* 1990), GP57 and GP65 glycoproteins (Satoh *et al.* 1986; Satoh & Fujii 1988).

The pattern and quantity of storage protein accumulation is also influenced by the maturation protocol used (Misra 1994; Sharma & Thorpe 1995). Misra *et al.* (1993) notes

that while white spruce somatic embryos matured on a medium containing ABA and high osmolarity, they failed to accumulate some of the major matrix and crystalloid proteins present in mature zygotic embryos. However, when embryos were matured on a medium containing 7.5% PEG-4000 plus 16μM ABA a wider range of undefined proteins were produced.

2.3.1.3 Extra-cellular proteins

Extracellular proteins have been isolated from media containing embryogenic cells (LoSchiavo *et al.* 1990; Gavish *et al.* 1992; Coutos-Thevenot *et al.* 1992). Akazawa and Hara-Nishimura (1985) briefly reviewed the extracellular polypeptides secreted into the plant suspension culture medium.

The precise role of extracellular proteins in somatic embryogenesis has not been elucidated. However, LoSchiavo *et al.* (1990) suggest some extracellular glycoproteins may be required to initiate the transition from globular to heart stage embryo in carrot cultures. Studies conducted with temperature sensitive carrot cell line variants have revealed that the function of these extracellular glycoproteins may be dependent on carrot modification of their oligosaccharide side chains. The accumulation of extracellular glycoproteins in the medium of embryogenic cultures have coincided with the removal of auxin from the medium. Further, the pattern of extracellular protein accumulation may be influenced by the age of the culture and the presence of auxin. The extracellular proteins identified by Coutos-Thevenot *et al.* (1992) blocked embryo development beyond the heart stage. This inhibitory effect of extracellular glycoproteins has also been demonstrated by Gavish *et al.* (1992). A family of extracellular glycoproteins (MW 53-57 KDa) secreted into the medium of citrus suspension cultures inhibited the transition of proembryo masses to globular stage embryos. These extracellular glycoproteins were released from the PEMs and, like other glycoproteins identified in carrot cell cultures and cell cultures of other plant species, are thought to play a regulatory role in somatic embryogenesis (LoSchiavo *et al.* 1990; Coutos-Thevenot *et al.* 1992). Other extracellular proteins also play a role in cell wall metabolism (de Vries *et al.* 1988), defense activities (Satoh & Fujii 1988), protease activity (Carlberg *et al.* 1984; O'Neill & Scott 1987), and

transformation of nucleotides into permeable compounds for membranes and incorporation into DNA (Ciarrocchi *et al.* 1981).

2.3.1.4 Storage carbohydrates

In addition to lipid and protein reserves, maturing somatic embryos also accumulate carbohydrates. The starch content of somatic embryos normally increases during maturation and is higher than in zygotic embryos (Lindsey & Topping 1993). The higher level of starch found in somatic embryos may be attributed to an inefficiency in carbohydrate conversion to lipids and proteins (Sharma & Thorpe 1995). As with lipid and protein reserves, the maturation protocol also influences the type, accumulation and conversion of carbohydrate reserves in the maturing embryo. Further, Thanh *et al.* (1985) observed that oligosaccharins (oligosaccharides with regulatory activity) induced morphogenesis in tobacco explants at concentrations of 10^{-8} to 10^{-9} M .

2.3.2 Molecular events

It is estimated that between 20 000-30 000 genes are expressed during embryogenesis (Goldberg *et al.* 1989). Some genes specific to early-stage carrot embryogenesis have been isolated and cloned (Komamine & Kawahara 1993; Dong & Dunstan 1996).

Investigations into the molecular events occurring after wounding of potato tubers reveal significant changes in various mRNA levels occur (Shirras & Northcote 1984; Logemann *et al.* 1988). These changes involve either transcription of genes encoding proteins that were detected only in wounded tissue, or suppressed (or reduced) transcription of other key genes. Although these studies did not involve somatic embryogenesis, a good indication is given of the molecular changes that can occur when plant tissues are exposed to the various stress inducing factors used during somatic embryogenesis.

Few embryo specific gene products have been identified so far. As embryogenic cells are metabolically very active, it is expected that gene products required for activities such as respiration, cell wall synthesis and cell division would be found in abundance. However,

as similar gene activity would also be expected in meristematic cells and other actively dividing cells, these genes are not specific to embryogenesis. Techniques such as gene trapping, *in situ* hybridisation and the utilisation of embryo-lethal mutants have been used to investigate molecular changes occurring during the embryogenic process (reviewed in Lindsey & Topping 1993).

Factors controlling the expression of embryo-specific genes are still unclear. The answer may lie within the cells themselves. Fernandez *et al.* (1991) suggest the pattern of cell development of embryogenic cells may influence their pattern of gene expression. Alternatively, one may argue that it is the *position* of the cells within the embryo that influences their gene expression pattern.

2.4 SUMMARY OF OBJECTIVES FOR SECTION 2

Some of the major problems associated with somatic embryogenesis include the initiation of embryogenic calli, embryo development, maturation and regeneration, and the transfer of regenerated plantlets to a greenhouse environment.

The following chapters of this section focused on the production and acclimatisation of somatic embryos for six asparagus cultivars (a more detailed outline of the objectives was given in Chapter 1). Factors such as plant genotype, the type and concentration of plant growth regulators (PGRs) influencing embryo induction, development and conversion were evaluated. Protocols for callus induction, somatic embryo development and conversion were also developed. Plantlets regenerated during the study were acclimatised and transferred to the greenhouse. Protocols were also developed to enhance survival of acclimatised plantlets.

CHAPTER THREE

Asparagus somatic embryogenesis: 1. Induction of embryogenic calli using varying plant growth regulator concentrations

Abstract

Six asparagus cultivars (Aspiring, Karapiro, Pacifica, Turoa, Syn4, and UC 157) were investigated for their potential to produce embryogenic calli when treated with different plant growth regulator (PGR) concentrations. Callus induction was promoted on Murashige and Skoog (MS) media containing 3% sucrose, 1% agar and one of the following PGRs: 0, 0.1, 0.3, 1, 3, 10, 20, and 30 μM 2,4-D and 1.0 μM NAA/ 0.1 μM Kinetin). Plant genotype, PGR concentration and length of time in culture significantly influenced both the number of explants producing calli and the type of calli developing from explants. Plant growth regulator concentrations of 0.3, 1, 3, and 10 μM 2,4-D and 1.0 μM NAA/ 0.1 μM Kinetin produced embryogenic calli for all cultivars and were, therefore, selected for further studies on somatic embryo development and regeneration.

Keywords

Asparagus officinalis, 2,4-dichlorophenoxyacetic acid (2,4-D), Napthaleneacetic acid (NAA), Kinetin (Kn), somatic embyrogenesis, callus induction

3.1 EXPERIMENT 1

3.1.1 INTRODUCTION

Recent advances in asparagus micropropagation (Chin 1982; Desjardins *et al.* 1987; Khunachak *et al.* 1987; Conner & Falloon 1993) have increased the potential of using this technique to establish commercial fields with high yielding uniform plants (Abernethy & Conner 1992; McCormick & Franklin 1990). Several micropropagation techniques investigated for the production of asparagus clones include shoot tip culture (Loo 1945; Morel & Wetmore 1951; Yang & Clore 1973, 1974); meristem tip culture (Murashige *et al.* 1972; Desjardins 1992); adventitious shoot culture (Takatori *et al.* 1968); organogenesis (Steward & Mapes 1971; Bui-Dang-Ha 1975; Dan & Stephens 1991); protoplasts culture (Mukhopadhyay & Desjardins 1994a and 1994b; May & Sink 1996; Ziauddin et al. 1996); anther culture (Wolyn & Feng 1993; Falavigna *et al.* 1996) and somatic embryogenesis (Wilmar & Hellendroorn 1968; Reuther 1996). Of the many techniques associated with large scale propagation of asparagus clones, somatic embryogenesis offers several advantages that have a direct impact on reducing the overall cost of clones. These advantages include reduced labour costs, a reduction in time plantlets spend in culture and adaptability to large-scale bioreactor systems.

Since the early work conducted on asparagus somatic embryogenesis (Wilmar & Hellendroorn 1968), research has been undertaken to improve the efficiency of embryo development and plant regeneration. The following is a brief summary of some of these approaches:

- determination of the appropriate carbohydrate source and concentration required for efficient induction of somatic embyros (Levi & Sink 1990);
- development of a protocol for the production of somatic embryos from protoplasts (Kunitake & Mii 1990);
- the proliferation of non-vitrified somatic embryos by utilising aseptic ventilative filters plus high (1%) gelling agent concentration in the media (Saito *et al.* 1991);

- determination of the influence of explant source and PGRs on the morphology and development of somatic embryos (Ghosh & Sen 1991; Levi & Sink 1991a and 1991b);
- development of protocols for embryo induction from anther culture to recover supermales for breeding programs (Falavigna *et al.* 1996; and
- production of somatic embryos in suspension cultures in bioreactors (Reuther 1996).

The potential for plant cultivars to produce embryogenic calli and somatic embryos is influenced by the genotype, the type and concentration of PGRs used (Borkird *et al.* 1986; Levi & Sink 1991b). Therefore, it is difficult to develop generic somatic embryogenesis protocols for all cultivars, although auxins such as 2,4-D, NAA and kinetin have been used to initiate the development of embryogenic callus on explants from plant species (Maheswaran & Williams 1984; Dudits *et al.* 1991) including asparagus (Kar & Sen 1985).

The effect of PGR concentrations on the development of embryogenic calli from six asparagus genotypes are reported in this study.

3.1.2 MATERIALS AND METHODS

3.1.2.1 Plant material

Three cultivars, Pacifica (PC), UC157 (UC) and Syn4 (SN), were maintained at the greenhouse units, Massey University, Palmerston North, New Zealand. Both 'UC' and 'SN' were established from seed while 'PC' was established from clonal material. Cultivars, Aspiring (ASP), Karapiro (KP) and Turoa (TU) were obtained and maintained as tissue-cultured liners from Aspara Pacific, New Zealand. Young, actively growing spears were collected from greenhouse grown cultivars and surface sterilised in 10% v/v commercial bleach (Janola®, 42g/l sodium hypochlorite) containing 2 drops Tween 20 per liter, for 15 minutes. This was followed by 3 washes (5 minutes each) in sterile distilled water. Explants from tissue-cultured material were used directly. Explants were divided

into 5-10 mm sections and transferred to 90 mm disposable plastic petri dishes containing 10-15 ml callus induction medium.

3.1.2.2 Callus induction

To determine the embryogenic potential of each cultivar, and the optimum plant growth regulator concentration for the production of embryogenic calli, explant sections were grown on callus induction (CI) media. These media contained Murashige and Skoog (MS) nutrients (Murashige & Skoog 1962) supplemented with one of eight 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations from 0, 0.1, 0.3, 1.0, 3.0, 10, 20, and 30 μM or a combination of 1.0 μM NAA/ 0.1 μM Kinetin. All CI media formulations contained 3% sucrose and 1% agar and the pH was adjusted to 5.8 prior to autoclaving (1.05 kg/cm^2 for 15 min). Three petri dishes containing five explant pieces were used per cultivar. Each petri dish was used as a replicate and kept in one of the following locations: two growth chambers (Skope™) and a larger controlled temperature room. All cultures were incubated in the dark at 25 ± 1°C. Explants with callus were assessed over a 21 day period.

3.1.2.3 Statistical analysis

A randomised complete block design was used with each growth chamber used as a replicate. A three factor (time, cultivar and PGR treatment) analysis of variance was performed using MSTAT-C (1989). Treatment means were separated using Fisher's least significant difference (LSD) at the 5% significance level as described by Steele & Torrie (1980).

3.1.3.4 Colour chart

A reference colour chart for all colours used in this and subsequent sections is found in Appendix Plate 3.0.

3.1.3 RESULTS

3.1.3.1 Callus induction

Callus formed on the cut surface of explants within 3 weeks. The average number of explants producing callus over a 21-day period, for all cultivars at each PGR treatment, is shown in Table 3.1. The main effects of time, cultivar and PGR treatment were all highly significant ($P = 0.0001$). The interaction of all three main effects were also significant ($P = 0.001$). The PGR concentration also significantly ($P = 0.0001$) influenced the number of explants producing callus. The general trend for all cultivars showed that as 2,4-D concentration increased less callus was produced on explants (Figure 3.1). The growth inhibitory effect of 2,4-D concentrations 10 μM and greater was more noticeable for explants derived from *in vitro* maintained cultivars than greenhouse grown stock plants. The optimum PGR concentration for callus initiated on explants was influenced by plant genotype and source of explant. For example, the optimum PGR concentrations for callus production on 'ASP', 'KP', and 'TU' explants (from *in vitro* maintained stocks) were 0, 0.1, and 0.3 μM 2,4-D. These PGR treatments resulted in the production of callus on approximately 3-5 explants by Day 21. In contrast, 4-5 'PC', 'SN', and 'UC' explants (from greenhouse maintained stocks) produced callus within 4 days of transfer onto media containing all PGR treatments except 20 and 30 μM. Callus production on 1.0 μM NAA/0.1μM Kinetin and 1.0 μM 2,4-D treatments was similar for all cultivars studied.

The effectiveness of PGRs to induce formation of callus on explants was very cultivar dependent. Treatments of 10, 20, and 30 μM 2,4-D generally produced less callus for all cultivars, except for cultivar ASP where treatments 0 and 10 μM 2,4-D and 1.0 μM NAA/0.1μM Kinetin did not induce callus development within 21 days. Most calli were produced on 0 and 0.3 μM 2,4-D for all cultivars, except ASP. Normally, explants produced callus between day 4 and 10 and by day 17 explants of most cultivars had produced callus (Table 3.1). Marked cultivar differences in the number of explants producing callus, for each PGR treatment were also noted, the exceptions being cultivars TU and ASP. The order of performance of each cultivar from the most explants with

callus to the least is: PC, UC, JG, KP, TU, and ASP. Explants collected from greenhouse grown plant material ('PC', 'SN', and 'UC') produced more callus within 21 days than explants derived from tissue culture maintained cultivars ('ASP', 'KP', and 'TU'). Callus was evident on 'PC', 'SN', and 'UC' explants 4 days after transfer to callus induction medium, while callus production occurred on 'ASP', 'KP', and 'TU' explants from Day 10 (Table 3.1).

Greenhouse grown explants from 'PC', 'SN', and 'UC' were larger than those obtained from *in vitro* maintained stock. A lower percent (approximately 30%) of explants obtained from *in vitro* maintained stock plants produced calli within 21 days. The number of explants producing callus also varied within replicates. Explants maintained in the two controlled growth chambers in comparable treatments did not vary in explant callus production. However, explants kept in a larger growth room produced less callus. These observations were consistent for all cultivars.

3.1.3.2 Condition of explant

Prior to transfer to the culture media all explants were green in colour. The physical appearance of explants was affected both by the length of time in the culture and by the PGR treatment (Table 3.2). The appearance of explants is shown in Plate 3.1a Explants on basal CI media remained green over the 21-day period. Explants became chlorotic at higher PGR concentrations the longer they remained in culture. By day 14 explants on 2,4-D concentrations of 20 and 30 μM were yellow-brown in appearance; callus was not produced on these explants. Generally, more of the explants derived from tissue cultured stock of 'ASP', 'KP', and 'TU' were yellow-brown by day 10 than for greenhouse grown cultivars (PC, SN and UC).

3.1.3.3 Selection of embryogenic calli

Three weeks after growth on CI medium, calli were removed from explants and subcultured onto their respective media. Four weeks later, calli were scored for presence or absence of embryogenic calli (Table 3.3). Only embryogenic calli were selected for further studies. Embryogenic potential of calli was assessed by evidence of differentiation

(globular embryos, organogenesis or root extensions from calli masses) (Plate 3.1b). Several calli types were observed, ranging from white-crystalline to yellow-mucilaginous (Table 3.3). Calli developed on basal media were typically white-crystalline for all cultivars except for cultivar TU which produced calli tan in appearance. Calli developed on explants grown on most media treatments were variable in appearance for each cultivar, while calli developed on higher 2,4-D concentration (10, 20, and 30 μM) were usually yellow in colour and mucilaginous for all cultivars.

Table 3.1 Effect of PGR treatment on the number of explants producing callus for each asparagus cultivar. Data are the means of 3 replicates. SEM = 0.1.

Cultivar	Treatment	Days after subculture					
	µM 2,4-D	1	4	10	14	17	21
Aspiring	0	0	0	0	0	0	0.7
	0.1	0	0	1.7	1.7	2.7	2.7
	0.3	0	0	1	1.7	2.7	3.3
	1	0	0	0	0	0	0
	3	0	0	0.7	0.7	0.7	0.7
	10	0	0	0	0	0	0
	20	0	0	0	0	0	1.3
	30	0	0	0	0	0	0.7
	NAA/Kn	0	0	0	0	0	0
Karapiro	0	0	0	4	4.7	5	5
	0.1	0	0	4.3	4.3	5	5
	0.3	0	0	4.3	5	5	5
	1	0	0	1	1	1	1.7
	3	0	0	0	0	0	0.7
	10	0	0	0	0	0	0.7
	20	0	0	0	0	0	0
	30	0	0	0	0	0	0
	NAA/Kn	0	0	0.7	1	1	1
Turoa	0	0	0	0.7	2.3	2.7	3.7
	0.1	0	0	0.7	1.7	2.7	3.7
	0.3	0	0	1.3	2.7	3	3.7
	1	0	0	0.7	1	1	1
	3	0	0	0	0.7	1	1
	10	0	0	0	0	0	0
	20	0	0	0	0	0	0
	30	0	0	0	0	0	0
	NAA/Kn	0	0	0.3	1	1.3	1.3
Pacifica	0	0	0.7	5	5	5	5
	0.1	0	0.7	4.7	4.7	5	5
	0.3	0	1.7	5	5	5	5
	1	0	0	4	4.7	5	5
	3	0	0	2.7	4	4.3	4.7
	10	0	0	3	3.7	4.7	5
	20	0	0	1.7	2	3.7	3.7
	30	0	0	1	1	1.7	1.7
	NAA/Kn	0	0	4.3	5	5	5
Syn4	0	0	0	3.7	4.7	4.7	5
	0.1	0	0	4.7	4.7	5	5
	0.3	0	0.7	5	5	5	5
	1	0	0	5	5	5	5
	3	0	0	4	4.3	4.3	4.3
	10	0	0	1	1.7	3.3	3.3
	20	0	0	0.7	1	2	2
	30	0	0	0.7	1	1	1
	NAA/Kn	0	0	4.7	5	5	5
UC157	0	0	0	5	5	5	5
	0.1	0	0	5	5	5	5
	0.3	0	0	5	5	5	5
	1	0	0	5	5	5	5
	3	0	0	4	5	5	5
	10	0	0	2	4.3	4.3	4.3
	20	0	0	1.7	1.7	1.7	1.7
	30	0	0	0	0	0	1
	NAA/Kn	0	0	4.7	5	5	5

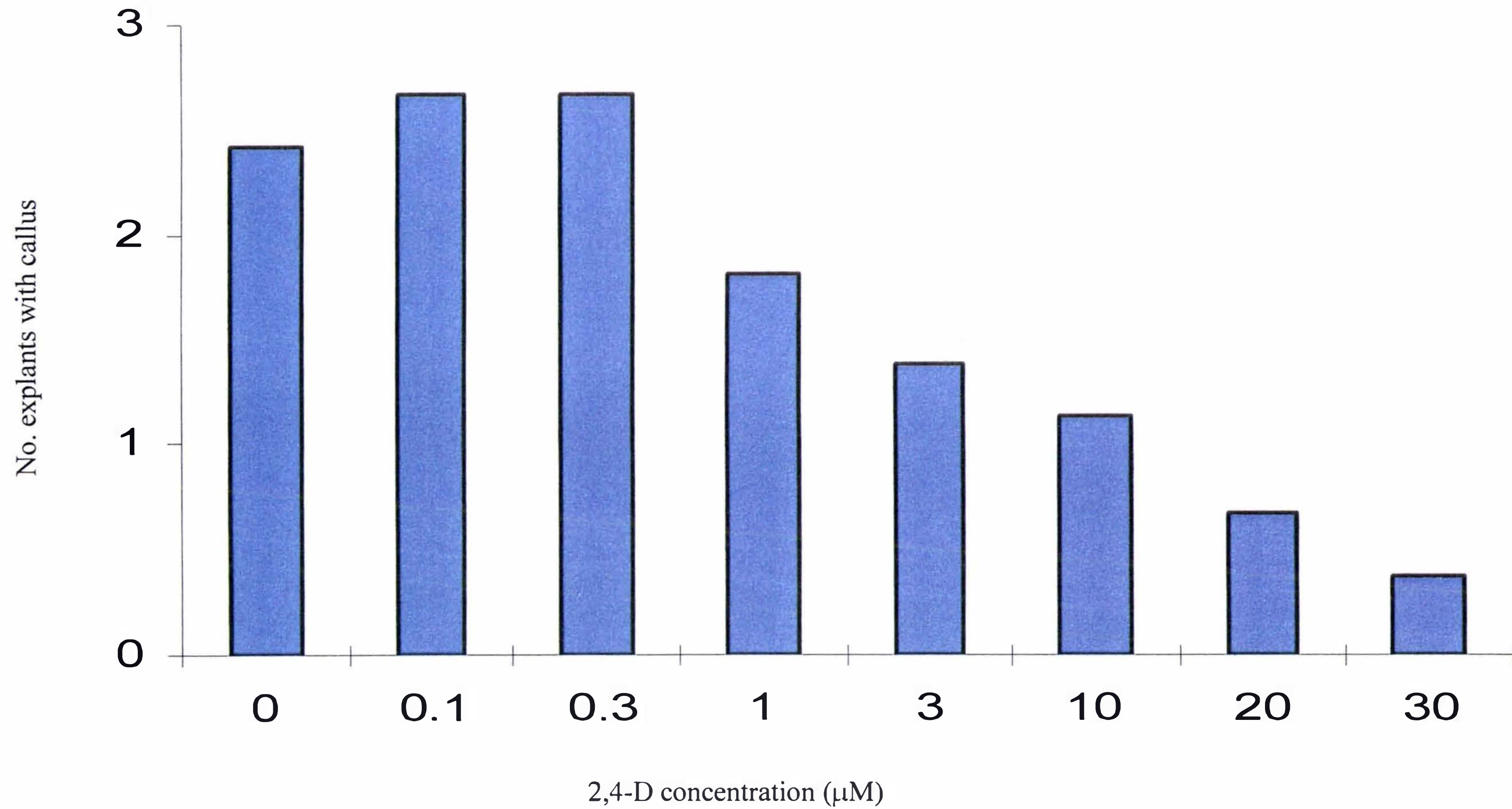


Figure 3.1 Plant growth regulator treatment effect on the number of explants producing calli. Bars represent the mean of all replicates on day 21 for all asparagus cultivars. SEM= 0.1, n=90.

Table 3.2 Morphology of explants grown on callus induction medium for 3 weeks. Key to table symbols: 'g' - green, 'gy' - green-yellow, 'yb' - yellow-brown and 'b' - brown.

Cultivar	Treatment	Days after subculture					
	μM 2,4-D	1	4	10	14	17	21
Aspiring	0	g	g	gy	gy	gy	gy
	0.1	g	g	gy	gy	gy	gy
	0.3	g	g	gy	gy	gy	gy
	1	g	g	yb	yb	yb	yb
	3	g	g	yb	yb	yb	yb
	10	g	g	yb	b	b	b
	20	g	g	yb	yb	yb	b
	30	g	g	yb	yb	yb	yb
	NAA/Kn	g	g	gy	gy	gy	gy
Karapiro	0	g	g	g	g	gy	gy
	0.1	g	g	gy	gy	gy	gy
	0.3	g	g	gy	gy	gy	gy
	1	g	g	gy	gy	gy	yb
	3	g	g	b	b	b	b
	10	g	g	yb	yb	yb	yb
	20	g	g	yb	yb	yb	yb
	30	g	g	gy	gy	yb	yb
	NAA/Kn	g	g	gy	gy	gy	gy
Turoa	0	g	g	gy	gy	gy	gy
	0.1	g	g	gy	gy	gy	gy
	0.3	g	g	gy	gy	gy	gy
	1	g	g	gy	gy	gy	gy
	3	g	g	gy	yb	yb	yb
	10	g	g	yb	yb	yb	yb
	20	g	g	gy	yb	yb	yb
	30	g	g	gy	yb	yb	yb
	NAA/Kn	g	g	gy	gy	gy	gy
Pacifica	0	g	g	g	g	gy	gy
	0.1	g	g	g	gy	gy	gy
	0.3	g	g	g	gy	gy	gy
	1	g	g	gy	gy	gy	gy
	3	g	g	gy	gy	gy	gy
	10	g	g	gy	gy	gy	gy
	20	g	g	gy	gy	gy	gy
	30	g	g	gy	gy	gy	gy
	NAA/Kn	g	g	g	g	gy	gy
Syn4	0	g	g	g	gy	gy	gy
	0.1	g	g	g	gy	gy	gy
	0.3	g	g	g	gy	gy	gy
	1	g	g	gy	gy	gy	yb
	3	g	g	gy	gy	gy	yb
	10	g	g	gy	gy	gy	b
	20	g	g	gy	gy	gy	yb
	30	g	g	gy	gy	gy	yb
	NAA/Kn	g	g	gy	gy	gy	yb
UC157	0	g	g	g	g	gy	gy
	0.1	g	g	g	g	gy	gy
	0.3	g	g	g	g	gy	gy
	1	g	g	gy	gy	gy	yb
	3	g	g	gy	gy	gy	yb
	10	g	g	gy	gy	gy	b
	20	g	g	gy	gy	gy	yb
	30	g	g	gy	gy	gy	yb
	NAA/Kn	g	g	gy	gy	gy	yb

Table 3.3 Description of callus phenotypes produced on CI media for all cultivars three months after initiation. Key to table symbols: '+' indicates presence of embryogenic callus, '-' indicates no embryogenic callus. 'B' brown, 'C' compact, 'E embryo development, 'F' friable, 'L' loose, 'M' mucilagenous, 'T' tan, 'R' root extensions, 'W' white, 'X' crystalline, 'Y' yellow.

Plant growth	Cultivar											
regulator	Apiring		Karapiro		Pacifica		Syn4		Turoa		UC157	
μM 2,4-D												
0	-	WX	-	WX	-	WX	-	WX	-	TC	-	WX
0.1	-	TC	-	YX	+	WX	-	TC	-	TC	-	WX
0.3	-	WX	+	YC	+	YXL	+	YX	+	TL	-	WXC
1	+	Y	-	TC	+	YF	-	WX	+	TF	+	YX
3	+	YM	+	WX	+	Y	+	YWE	+	YM	+	YX
10	-	WM	+	YX	+	YM	+	YX	-	YM	+	YX
20	+	YM	-	YM	-	YM	+	WX	-	YM	-	WX
30	-	YM	-	YM	-	YM	-	BWX	-	YM	-	YM
NAA/Kn	+	WC	-	WC	+	YCR	+	YC	+	TF	+	YCR

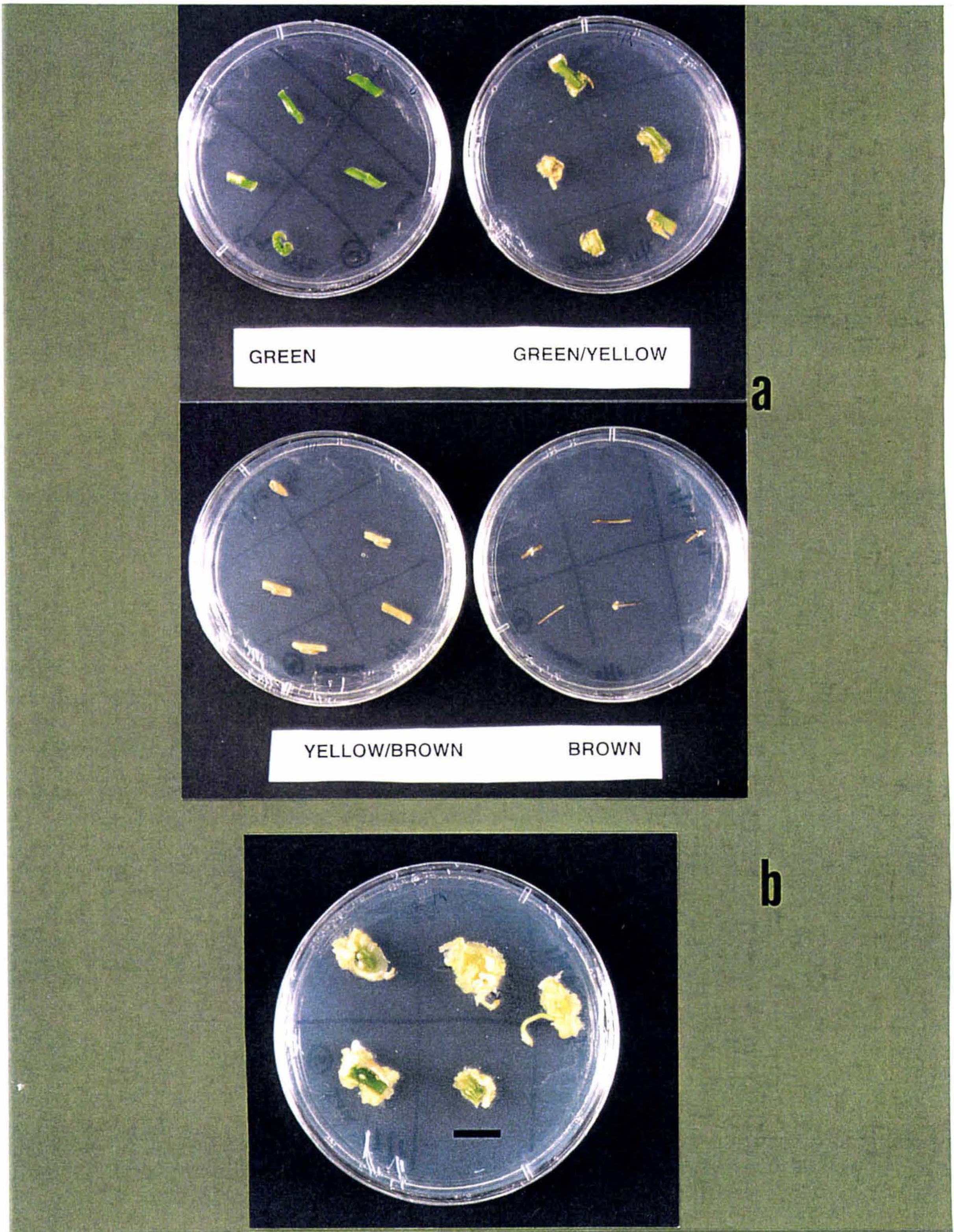


Plate 3.1 Growth of explants on callus induction medium (CI)

a. Colour of explants 10 days after inoculation onto CI medium.
b. Explants with embryogenic calli 21 days after inoculation onto CI media. Developing embryos and shoot growth evident.

3.1.4 DISCUSSION

Plant growth regulator treatment, source of explant (greenhouse vs. *in vitro*), plant genotype, and time significantly influenced the number of explants producing calli. The reduced number of explants producing calli with increasing PGR concentration may be due to the phytotoxic effect developing at higher concentrations. 2,4-dichlorophenoxyacetic acid, a potent auxin, at high concentrations is herbicidal and at lower concentrations would inhibit plant growth. Phytotoxicity was evident in the appearance of explants grown on CI media containing high 2,4-D concentrations where explants became chlorotic and died. Another factor contributing to the chlorotic condition of explants is the length of time they were removed from the mother plant. However, this time effect is expected to be minimal as explants were placed on growth media containing nutrients and a carbohydrate source to promote growth and development. In addition, explants grown on media without PGRs remained green.

Although calli were produced on explants at most PGR treatments, only treatments 0.3, 1.0, 3.0 and 10 µM 2,4-D and a combination of 1.0 µM NAA/ 0.1 µM Kinetin produced calli for all cultivars. More importantly, these five PGR treatments produced embryogenic calli that could be used in further studies on somatic embryo development.

Several factors influenced the initiation of embryogenic calli in asparagus. Genotype, PGR type and concentration are known to influence both the frequency of embryogenic calli development from explants and the development of asparagus somatic embryos from calli (Ghosh & Sen 1992; May & Sink 1996). The present results are consistent with these reports. Further, it is shown that the growth environment of the mother plant also influences the frequency of callus development on explants. Additional factors, such as developmental and physiological stage of development of explants, plus the growth environment of explant source, can also influence the efficiency of embryogenic calli development (Litz & Conover 1982; Monmarson *et al.* 1995). These factors could explain some of the results obtained in this study, in particular the difference in performance of explants collected from different sources.

3.1.5 SUMMARY

Plant genotype, PGR type and concentration, and length of time in culture influenced both the initiation of calli and the type of callus produced on explants. High PGR concentrations inhibited callus development. Concentrations of 0.3, 1.0, 3.0, and 10 μM and 1.0 μM NAA/ 0.1 μM Kinetin produced embryogenic calli in each cultivar.

Asparagus somatic embryogenesis: 2. Somatic embryo development, maturation and regeneration

Abstract

Several somatic embryo induction, maturation and regeneration protocols were investigated for their effectiveness to promote embryo development from embryogenic calli developed from three different asparagus genotypes. The three cultivars used in this experiment were Aspiring (ASP), Karapiro (KP) and Pacifica (PC). The following sequence was found to be most effective in producing complete plantlets from embryogenic calli: callus induction (CI) on Murashige and Skoog (MS) media containing 3% sucrose, 1% agar and either of 1.0, 3.0 and 10 μ 2,4-D, followed by transfer onto liquid embryo induction media (EI) containing MS + 6% sucrose and finally regeneration on regeneration media (Rg4) containing MS + 0.2 g/l glutamine + 3% sucrose + 1% agar. Globular embryos were produced by all cultivars; however, only globular embryos developed from cultivar PC converted to plantlets. Treatment of 'PC' globular embryos at -15^{0}C (due to equipment failure) produced the highest percent converted plantlets (34 and 26% for 6-month-old embryogenic calli and 1 year-old embryogenic suspension cells respectively). Precocious germination, rhizogenesis, recurrent embryogenesis and organogenesis were common problems encountered. Further studies are required to understand the molecular and biochemical events involved in triggering and switching off embryogenic gene expression.

Keywords

Asparagus officinalis, abscisic acid (ABA), osmotic potential, polyethylene glycol (PEG), embryo conversion, recurrent embryogenesis, rhizogenesis, organogenesis, precocious germination

3.2 EXPERIMENT 2

3.2.1 INTRODUCTION

Successful morphogenesis of somatic embryos from the globular embryo stage to complete plantlet is dependent on several factors including polar auxin transport (West & Harada 1993; Zimmerman 1993), plant species, genotype, age of plant cells (Attree *et al.* 1990a; Karunaratne *et al.* 1991), and the type and concentration of plant growth regulators used to initiate and maintain embryogenesis (Borkird *et al.* 1986; Smith & Krikorian 1990). Related research on asparagus somatic embryogenesis shows different PGRs can effectively enhance development of somatic embryos, Li and Wolyn (1995) showing that ancymidol, abscisic acid (ABA), uniconazole, and paclobutrazol could be used to enhance development and conversion of bipolar embryos of 'G447' asparagus cultivar.

Carbohydrates included in the embryo induction media can influence asparagus embryo development as transfer of globular embryos from embryo induction media containing high carbohydrate levels (4 - 10%) to media containing lower carbohydrate levels resulted in a 2 to 4 times increase in conversion of asparagus somatic embryos (Levi & Sink 1992). In an earlier study, Levi and Sink (1990) show that embryo conversion rates could also be increased by transferring globular embryos grown on media containing 5% sucrose to media containing 5% fructose.

After development of bipolar somatic embryos a period of maturation and developmental arrest occurs (West & Harada (1993). This period of maturation is an essential requirement for the accumulation of protein, carbohydrate and lipid reserves. Failure of somatic embryos to store these reserves may result in physiological disorders such as precocious germination, producing a large number of malformed embryos, and failure of embryos to convert to complete plantlets (Merkle *et al.* 1995; Reuther 1996). Abscisic acid and low osmotic potentials could play an important role in the accumulation of storage reserves required for somatic embryo maturation and conversion.

There are no detailed reports on embryo maturation studies conducted on asparagus embryogenesis, and no reports documenting the types of physiologically abnormal embryo development. For asparagus somatic embryogenesis to be a viable alternative for the commercial propagation of asparagus clones, more research is required to explore the factors influencing embryo maturation and conversion.

This study focused on the development of protocols for the development, maturation and conversion of somatic embryos derived from embryogenic calli of three asparagus clones. Examples of physiologically abnormal somatic embryos produced during the study are described.

3.2.2 MATERIALS AND METHODS

3.2.2.1 Plant material

Three clonal cultivars selected for this study were Aspiring (ASP), Karapiro (KP) and Pacifica (PC). Embryogenic calli from each cultivar were initiated during the callus induction experiment and used during the study.

3.2.2.2 Somatic embryo induction and maintenance of embryogenic calli

After 3 months subculture on callus induction media (MS media containing 0.3, 1.0, 3.0, and 10 µM 2,4-D and 1.0 µM NAA/0.1 µM Kinetin), embryogenic calli were transferred to liquid somatic embryo induction media. Four different embryo induction media were evaluated for their potential to support development of somatic embryos and to sustain long-term proliferation of embryogenic cells. Embryo induction (EI) media used were as follows: EI1 (MS + 6% sucrose); EI2 (MS + 3% sucrose); EI3 (MS + 0.05 µM 2,4-D + 6% sucrose); and EI4 (½MS + 1% sucrose). Three to five grams of embryogenic calli from each treatment were transferred into 250 ml flasks containing 30 ml EI media. Cultures were kept on a gyratory shaker at 90 rpm, 21°C and a 16-hr photoperiod with

fresh embryo induction media every three weeks. For each cultivar, 4 replicates of each treatment were used. A randomised complete block design was used with shelves on the shaker used as blocks. After 4 weeks, embryogenic cell cultures were evaluated for their ability to produce cells in suspension, the development of somatic embryos and the physical appearance of cells.

3.2.2.3 Growth of embryogenic cells

One hundred milligrams of embryogenic calli from each cultivar, for each PGR treatment, were transferred to 125 ml conical flasks containing 15 ml EI1 media. Embryogenic cell growth was maintained over a 30-day period with samples taken every 5 days. Four replicates of each treatment for each sample time were used. A randomised nested complete block design as a repeated measure analysis was used, with shelves on the gyratory shaker used as replicates. A three-factor (time, cultivar and PGR treatment) analysis of variance was performed using SAS Version 6.12 for Windows (SAS Institute Inc.). Treatment means in this and subsequent experiments were separated by LSD at the 5% significance level.

3.2.2.4 Somatic embryo development

As cultures maintained on EI1 medium produced somatic embryos in suspension, this medium was selected for further studies. Suspension cells from each cultivar were maintained on EI1 medium and subcultured every three weeks. Suspension cells of cultivar 'PC' initiated on MS medium containing 1.0 µM 2,4-D and maintained on EI media for 1 year were also evaluated in this study. At each subculture, samples of embryogenic suspensions were collected and their competence to produce somatic embryos evaluated after replating onto maturation media.

3.2.2.5 Somatic embryo maturation

A preliminary study identified the most effective medium for somatic embryo maturation. Liquid maturation media evaluated were as follows: M1 (MS + 10% glucose); M2 (MS + 2% sucrose); M3 (MS + 0.2 g/l glutamine + 3% sucrose); and, M4 (MS + 10 µM ABA +

2% sucrose); M3 (MS + 0.2 g/l glutamine + 3% sucrose); and, M4 (MS + 10 µM ABA + 2% sucrose). Combinations of abscisic acid (ABA) and polyethylene glycol (PEG) was also investigated [µM ABA: % PEG - (0,0); (10,0); (40:0); (0,5); (10,5); (40,3); (0,10); (10,10) and (40,10)]. Each media formulation was gelled with 1% agar. Media were evaluated for their capacity to mature globular stage somatic embryos. Embryogenic cells were grown on maturation media for two weeks. Incubation conditions were the same as for embryo induction. Four replicates of each treatment were arranged in a completely random design.

3.2.2.6 Embryo regeneration

Two weeks after growth on embryo maturation media, 100 to 200 mg of embryogenic cells were transferred to 90 mm disposable plastic petri dishes containing 10-15 ml embryo regeneration media. Regeneration media evaluated were as follows: Rgl (MS + 0.1 µM zeatin + 3% sucrose); Rg2 (MS + 3% sucrose); Rg3 (MS + 0.002 µM GA_3 + 3% sucrose); Rg4 (=M3) (MS + 0.2 g/l glutamine + 3% sucrose); Rg5 (MS + 0.1 µM NAA + 0.1 µM 2ip + 2% sucrose); and, Rg6 (Rg4 + 0.1 µM GA_3). All media were gelled with 1% agar. Embryogenic cells were kept on regeneration media in petri dishes until mature embryos (embryos 4-7 mm long with defined shoot and root apices (Levi & Sink 1992)) developed. Individual embryos were removed and transferred onto fresh regeneration media as they developed.

3.2.2.7 Maintenance of plantlets regenerated from somatic embryos

Converted somatic embryos (plantlets with shoot and root development) were transferred to 300 ml plastic tissue culture vessels containing 50 ml of Rg4 media and maintained at 21 ± 1^0C under cool white fluorescent lights at 30 µmol$m^{-2}s^{-1}$ and a 16-hr photoperiod.

3.2.2.8 Shock-treatment of long-term embryogenic suspension cells

Six-month-old embryogenic suspension cells from each cultivar, and one year old 'PC' embryogenic suspension cells grown in EI media were given a brief shock treatment for 1 hr. Treatments were as follows: MS basal media + 6 % sucrose containing one of the following PGRs, 10 and 100 μM BA, GA_3 and 2,4-D. Cells were washed three times with EI media and transferred to fresh EI media. Six-month-old embryogenic calli of all cultivars and 'PC' embryogenic suspension cells were inadvertently given a cold treatment at -15^{0}C for 3 hours when the incubator temperature controller failed. Cells surviving this treatment were transferred to fresh EI media and mature somatic embryos grown on Rg4 media.

3.2.2.9 Temperature treatment of globular somatic embryos

Three types of globular embryos (< 3 mm in diameter (Levi & Sink 1992)) identified in this study were used: embryos containing chlorophyll (chl+); chlorophyll deficient (chl-); and embryos with purple pigmentation (pur+). Globular embryos were transferred to Rg4 media and given the following cold treatments: 20, 15, 10, 5, 0, -5, -10, -15 and -20^{0}C. Temperatures of -5, -10 and -15^{0}C were obtained by mixing crushed ice with sodium chloride. For each treatment, 10 globular embryos were used. Because a limited number of globular embryos with purple pigment were available these were treated at 20, 0 and -10^{0}C only. Embryos were kept at each temperature for 1, 2 and 3 hrs and then transferred to 21 ± 1^{0}C for 4 weeks.

3.2.3 RESULTS

3.2.3.1 Somatic embryo development from embryogenic suspensions

Embryo development occurred on PGR-free EI1 and EI2 media and EI3 media containing 0.05 μM 2,4-D (Table 3.4). Somatic embryo development on EI3 medium was observed for cultivar PC only. Mature somatic embryos (4-7 mm long) were observed for cultures grown in PGR-free media (Plate 3.2a), while only early stage globular embryos (< 3 mm in diameter) were present in embryo induction media containing 2,4-D (Plate 3.2b). This

study and observations from preliminary studies (data not shown), show development of mature somatic embryos was better on EI1 medium. For this reason, EI1 medium was selected for further studies on embryo maturation and conversion. Embryogenic cell growth on EI3 and EI4 media was retarded and no somatic embryo development was observed. Cells developed on EI4 were brown in colour and eventually died after 2 subcultures. After 4 weeks of culture on embryo induction media, a heterogeneous population of freely suspended cells, globular stage embryos, mature embryos, and embryogenic cell masses was obtained (Plate 3.3a). Two morphologically distinct globular embryos were observed in culture media, a chlorophyll deficient embryo (Plate 3.3b) developed in all cultures for all PGR treatments while globular embryos, with chlorophyll (Plate 3.3c), were noted for 'ASP' and 'PC' cultures derived from calli initiated on 3 and 10 μM 2,4-D respectively. Approximately 10 and 90 percent of embryos recovered for 'PC' (10 μM 2,4-D callus induction treatment) and 'ASP' (3 μM 2,4-D), respectively, contained chlorophyll. Interestingly, 'PC' globular embryos with chlorophyll were produced only for the first subculture onto EI media, while chlorophyll deficient globular embryos were recovered from subsequent subcultures.

Somatic embryo development was arrested at the globular stage in all cultivars, including cultivar PC long-term embryogenic suspensions, grown on EI media. After 3 subcultures onto PGR-free EI media a homogeneous cell suspension of embryogenic cells was obtained. These cells were used for embryo maturation and conversion studies.

3.2.3.2 Growth of embryogenic cells in suspension culture

Embryogenic cell growth in embryo induction media was influenced by the PGR treatment used in the callus induction experiment, and significant ($P = 0.0001$) cultivar and time effects were observed. An increase in fresh weight of embryogenic calli transferred to EI medium was observed overtime for all cultivars at each PGR pretreatment (Figure 3.2). Calli from all cultivars initiated on 3.0 μM 2,4-D grew better when transferred to embryo induction media while the slowest growth was observed for calli initiated on 0.3 μM 2,4-D (Figure 3.3a). No significant difference in the growth rate

μM 2,4-D or 1.0 μM NAA/ 0.1 μM Kinetin was observed. No significant difference in the growth of calli from cultivars PC and ASP was noted. However, growth of 'KP' calli was slower than that observed for either 'PC' or 'ASP' (Figure 3.3b). No significant replication effects were observed for this study.

3.2.3.3 Long-term embryogenic cells

Embryogenic cells recovered from treatment at -15^{0}C for 3 hr produced early stage and mature globular embryos. Treatment of long-term embryogenic cells with BA, GA_3 and 2,4-D did not enhance proliferation of embryogenic cells or development of mature embryos.

Table 3.4 Somatic embryo development and morphology on embryo induction media. Plant growth regulator treatments 0.3, 1.0, 3 and 10 μM 2,4-D are indicated by 1, 2, 3 and 4. Treatment 1.0 μM NAA/ 0.1 μM Kinetin is indicated by 5. Key to table symbols: '+' indicates somatic embryo development, '-' indicates no embryos development. 'G' ,'M' and 'EM' indicates globular and mature embryos and embryogenic cell masses respectively.

Cultivar	EI1		EI2		EI3		EI4	
Aspiring								
1	+	G	-	EM	-	EM	-	EM
2	+	G	+	G	-	EM	-	EM
3	+	G/M	+	G	-	EM	-	EM
4	+	G/M	+	G/M	-	EM	-	EM
5	+	G	-	EM	-	EM	-	EM
Karapiro								
1	+	G	+	G	-	EM	-	EM
2	+	G	+	G	-	EM	-	EM
3	+	G	+	G	-	EM	-	EM
4	+	G	+	G	-	EM	-	EM
5	+	G	-	EM	-	EM	-	EM
Pacifica								
1	+	G	+	G	-	EM	-	EM
2	+	G	+	G	-	EM	-	EM
3	+	G/M	+	G/M	-	EM	-	EM
4	+	G/M	+	G/M	+	G/M	-	EM
5	+	G	+	G	-	EM	-	EM

3.2.3.4 Somatic embryo maturation

Several embryogenic cell lines developed for each cultivar failed to mature beyond either the globular or mature embryo stage. Precocious germination of embryos was also a common occurrence. Several maturation media were evaluated for their potential to solve these problems. Globular somatic embryos transferred from EI directly onto M3 media produced somatic embryos that developed to mature embryo stage for all cultivars. Cultivar PC somatic embryos, derived from calli initiated on 10 μM 2,4-D, also developed to the mature embryo stage on M2 medium.

3.2.3.5 Embryo regeneration

Complete embryo conversion, i.e. simultaneous development of root and shoot primordia, occurred on Rg4 media. Seventy-seven percent of plantlets regenerated in this study were from chl+ globular embryos, while 23 % were from chl- embryos. Regenerated somatic embryos were only recovered from 'PC' somatic embryos derived from embryogenic calli initiated on 3.0 and 10 μM 2,4-D and those embryogenic calli treated at -15^{0}C (Table 3.5). The highest embryo regeneration rate occurred from embryogenic cells treated at -15^{0}C, 34% and 26% for 'PC' (callus induction on 10 μM) and 'PC' (1 year-old suspension cells derived from calli initiated on 1.0 μM 2,4-D), respectively. Regeneration was observed 35 days after transfer of globular stage somatic embryos from EI1 or M3 media to Rg4 medium. Some developing somatic embryos aggregated together in clumps at the shoot end. Normally, more than one shoot grew from these clumps (Plate 3.4). Separation of intact somatic embryos at this stage was difficult. Development of somatic embryos from globular embryo to regenerated plantlet was characterised by the stages shown in Plate 3.5. Shoot development on some mature embryos (about 10%) occurred 3 months after evidence of root growth. In such cases, roots grew to approximately 5-10 mm before further growth ceased. Root growth continued only after shoot development commenced.

3.2.3.6 Effect of temperature on embryo conversion

As shown in Table 3.6 globular embryos containing chlorophyll responded better to temperature treatments than chlorophyll deficient embryos or those embryos containing purple pigments. Treatment of chl+ embryos at -5^{0}C, all times considered, resulted in the highest percent of regenerated plantlets (17%). The second highest percent embryo regeneration was noted for embryos treated at 0 and -20^{0}C, with a total of 13% conversion of globular embryos. Ten percent of chl- embryos, treated at -20^{0}C for 1 hour, produced plantlets and a total of 3% of these chl- embryos produced embryos. None of the pur embryos produced plantlets. Recurrent embryogenesis was observed on all pur embryos.

Table 3.5 Regeneration of somatic embryos maintained on Rg4 medium. '-' indicates no regeneration. Plant growth regulator treatments 0.3, 1.0, 3 and 10 µM 2,4-D are indicated by 1, 2, 3 and 4. Treatment 1.0 µM NAA/ 0.1 µM Kinetin is indicated by 5. Key to table symbols: '*' clumps of embryos with shoot development were not counted. 'a, b, and c' represents cultivar PC 1 year embryogenic suspension cultures, embryogenic calli treated at -15^{0}C and 1 year old suspension cells treated at -15^{0}C respectively.

Cultivar	Callus induction treatment	Maturation treatment	No. mature embryos	No. regenerated embryos		% regeneration
				Single	Clumps	
Aspiring						
	1	M3	-	-	-	-
	2	M3	-	-	-	-
	3	M3	-	-	-	-
	4	M3	10	0	0	0
	5	M3	-	-	-	-
Karapiro						
	1	M3	-	-	-	-
	2	M3	-	-	-	-
	3	M3	20	0	0	0
	4	M3	12	0	0	0
	5	M3	-	-	-	-
Pacifica						
	1	M3	70	0	0	0
	2	M3	-	-	-	-
	3	M3	85	0	0	0
	4	M2 & M3	1200	169	18*	11
	5	M3	0	0	0	0
> 1 yr[a]	2	M3	343	0	26*	*
F[b]	4	M2	85	29	0	34
F >1yr[c]	2	M3	156	41	0	26

Table 3.6 Plantlet regeneration from 'Aspiring' globular somatic embryos (derived from calli initiated on callus induction medium containing 3.0 μM 2,4-D) treated at different temperatures. Key to table symbols: 'r', 'rh' and 'n' indicates recurrent embryogenesis, rhizogenesis, and absence of embryos, respectively.

Temperature (⁰C)	Time (hrs)	Percent regenerated plantlets		
		chl+	chl-	pur+
20	1	0	0	0 r
	2	0	0	0 r
	3	0	0	0 r
	Total % conversion	0	0	0
15	1	0 r	0	n
	2	10 r	0	n
	3	0 r	0 r	n
	Total % conversion	3	0	n
10	1	0 r	0	n
	2	0 r	0	n
	3	20	0	n
	Total % conversion	6	0	n
5	1	0 r	0	n
	2	20	0	n
	3	0 r	0	n
	Total % conversion	6	0	n
0	1	0	0	0 r
	2	20	0	0 r
	3	20	0	0 r
	Total % conversion	13	0	0
-5	1	20 r	0	n
	2	20 r	0	n
	3	10	0	n
	Total % conversion	17	0	n
-10	1	0 r	0	0 r
	2	0 r	0 r	0 r
	3	0 r	0	0 r
	Total % conversion	0	0	0
-15	1	0 r	0	n
	2	10 r	0	n
	3	0 r	0 r	n
	Total % conversion	3	0	n
-20	1	10	0	n
	2	20 r	10 rh	n
	3	10 r	0	n
	Total % conversion	13	3	n

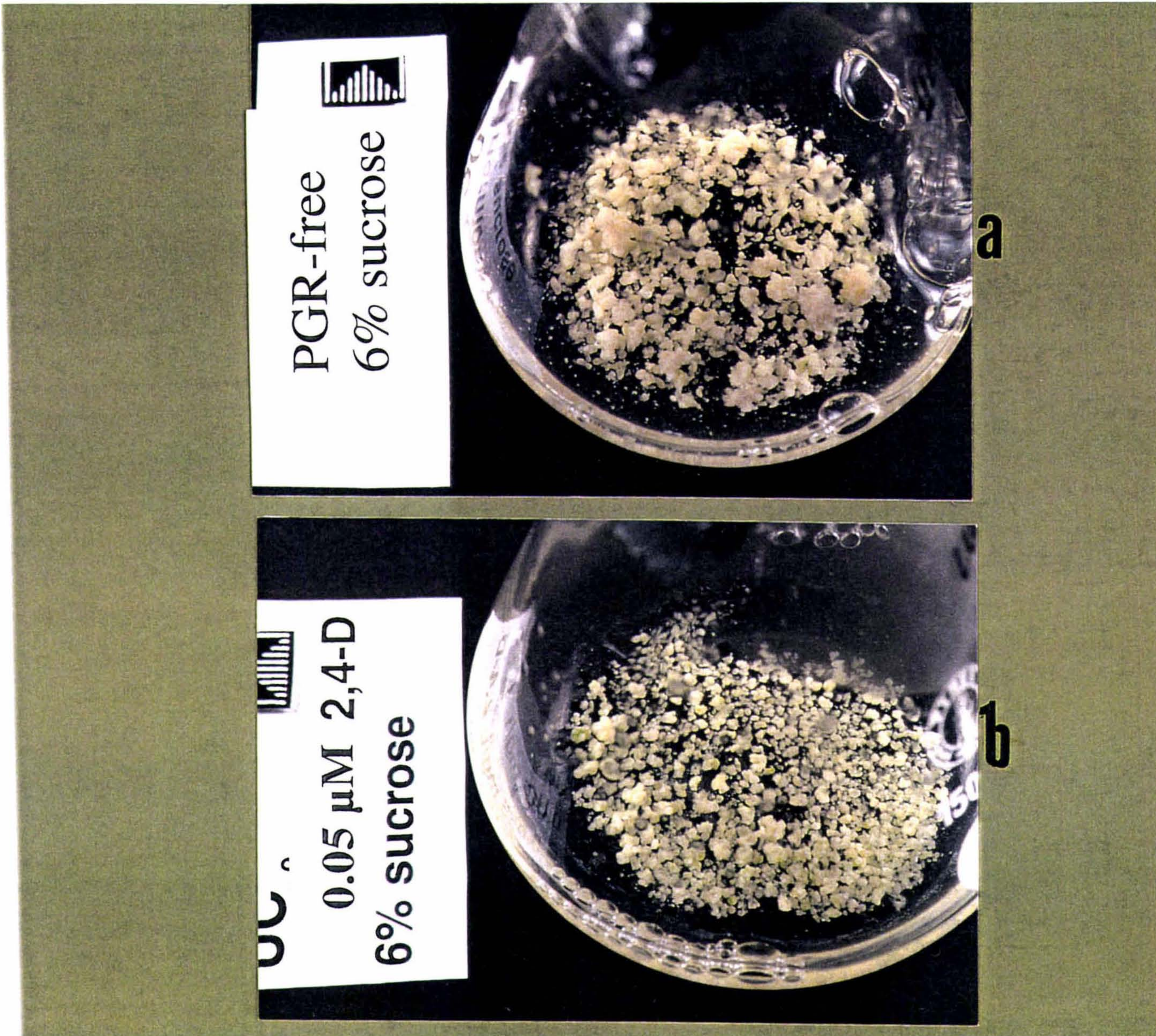


Plate 3.2 Somatic embryo development on embryo induction medium (MS + 6% sucrose)

a. Heterogeneous cell population of early and mature stage embryos. Scale = 1 cm
b. Embryo development on EI medium containing 2,4-D. The cell suspension contains mainly early stage globular embryos. Scale = 1 cm

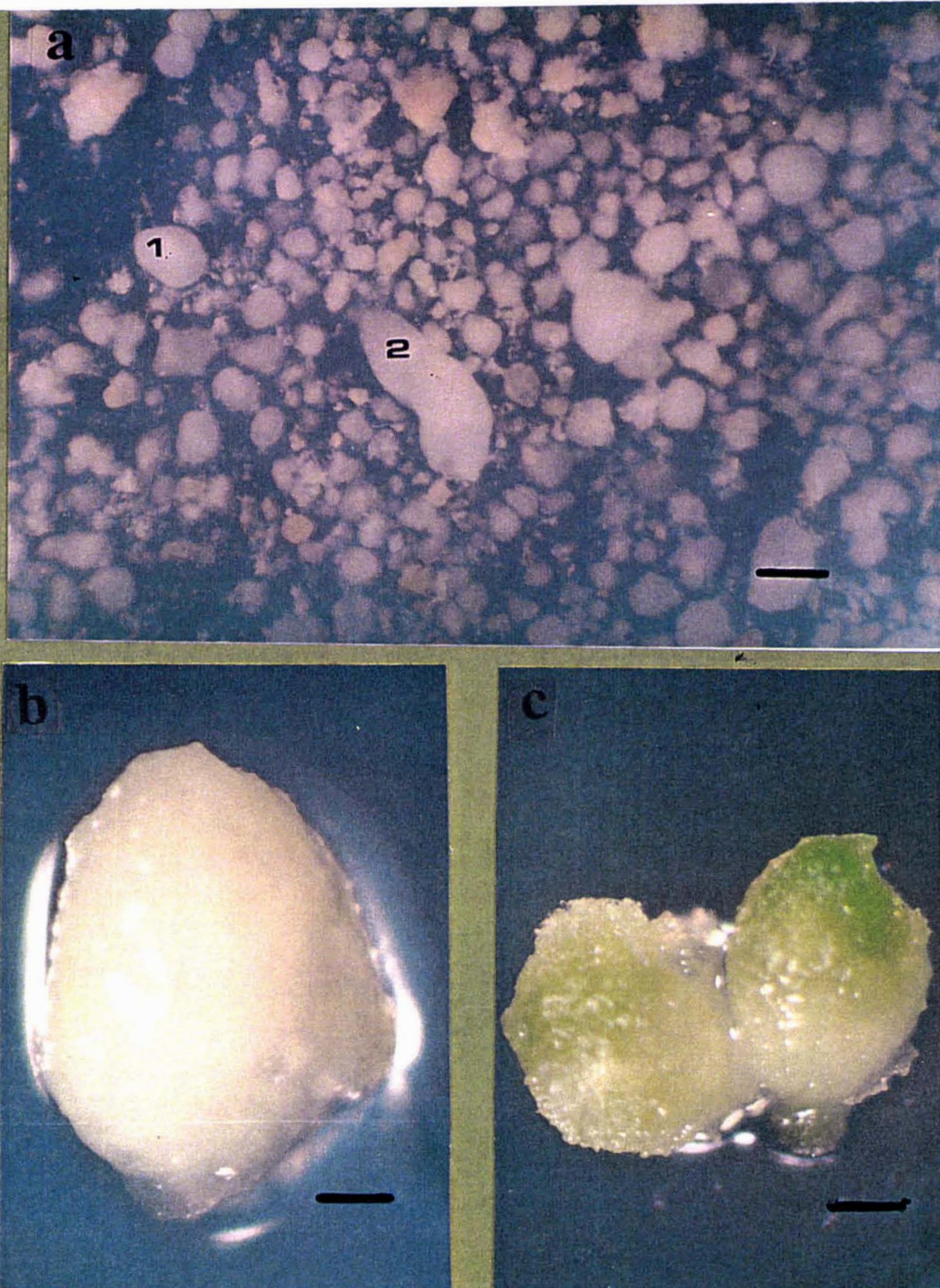


Plate 3.3 Somatic embryos

a. Heterogeneous cell population containing (1) early stage globular embryos (< 3 mm in diameter) and (2) mature embryos (4 - 7 mm long). Bar = 1.4 mm

b. Chlorophyll deficient globular embryo. Bar = 0.4 mm

c. Globular embryos with chlorophyll. Bar = 0.3 mm

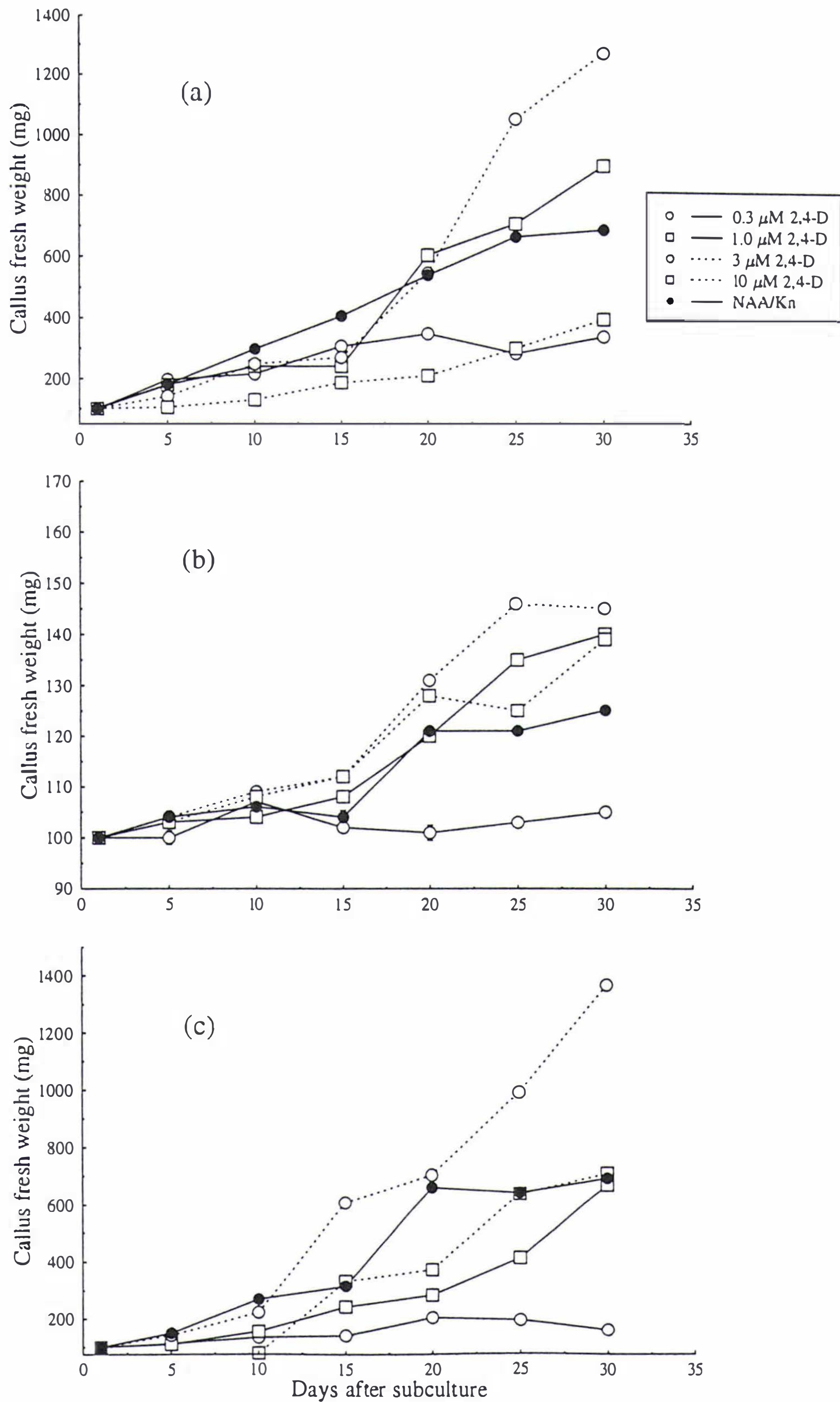


Figure 3.2 Growth of cultivars Aspiring (a), Karapiro (b) and Pacifica (c) embryogenic cells in EI medium. Each point represents the mean of 4 replicates. SEM ≤ 0.1, n=4.

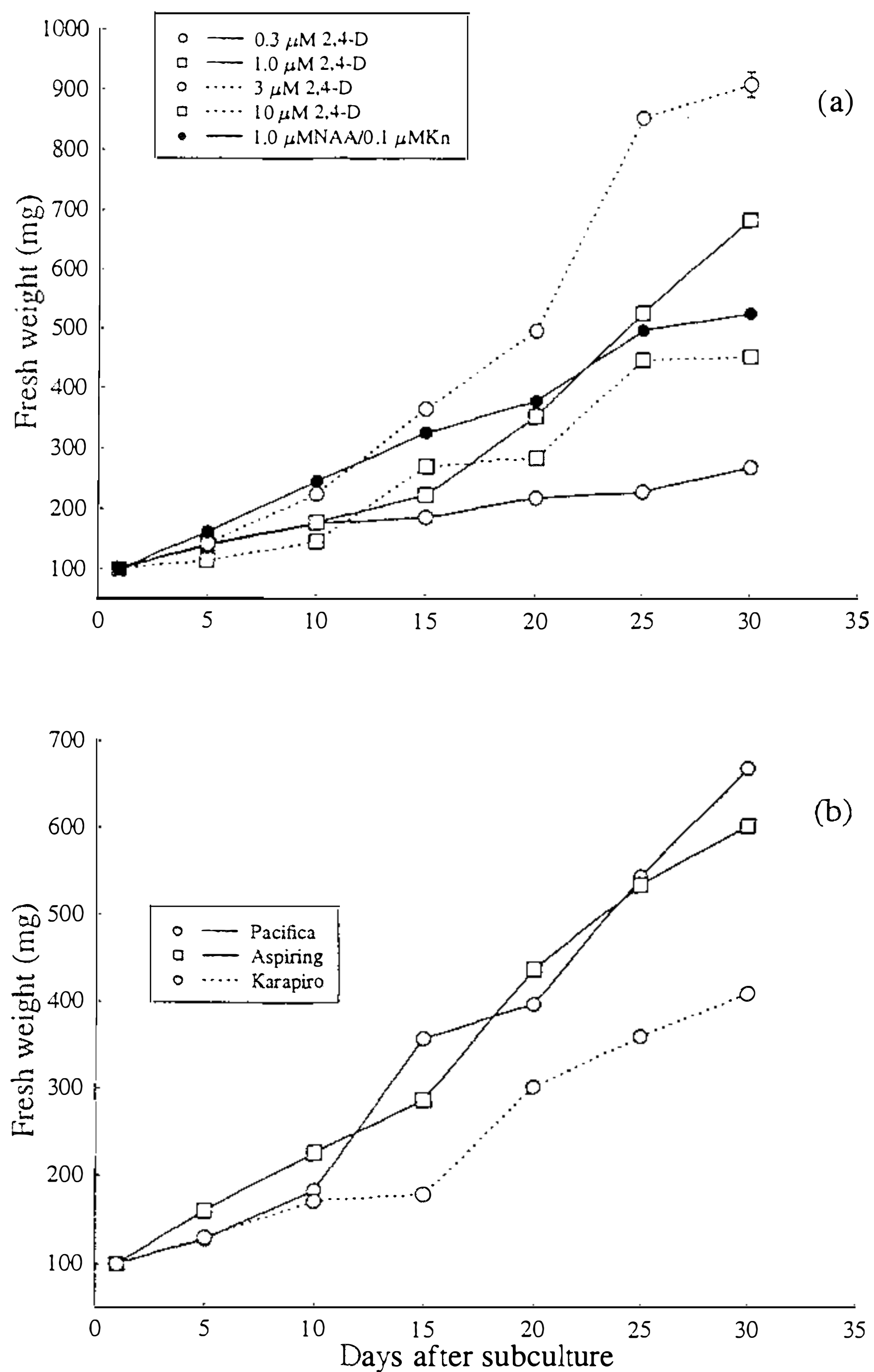


Figure 3.3 Asparagus embryogenic cell growth in EI1 medium (a) mean fresh weight of calli for cultivars Aspiring, Karapiro and Pacifica for each PGR treatment. (b) Growth of calli of each cultivar for all PGR treatments. SEM $\leq$ 0.1.

3.2.3.7 Abnormal physiological development of somatic embryos

Some common examples of abnormal somatic embryo development identified in this study are shown in Plates 3.6 - 3.8. Precocious germination was observed for all cultivars and was characterised by the development of the root primordia without the corresponding development of the shoot primordia (Plate 3.6a). Sometimes (5%) globular embryos failed to mature but instead developed as shown in Plate 3.6c & d. Recurrent embryogenesis was the most common problem identified for all cell lines developed from each cultivar at PGR treatments supporting somatic embryo development. Recurrent embryogenesis was characterised by the development of embryogenic calli along the developing embryo, particularly at the shoot end. Whenever recurrent embryogenesis was observed, further embryo development was inhibited. Recurrent embryogenesis occurring on matured somatic embryos is shown in Plate 3.7. Rhizogenesis was observed during the first subculture of embryogenic calli onto embryo induction media. Rhizogenesis was commonly observed either as a dense mass of roots extending from a central embryogenic cell mass or as single root extensions in suspension cultures Plate 3.8. Organogenesis was evident in some embryogenic cultures specifically those derived from cultivar ASP and TU initiated on medium containing 1.0 μM NAA/ 0.1μM Kinetin. Direct organogenesis was characterised by the growth of cladophylls (shoots) from embryogenic calli without any root development (Plate 3.8c).

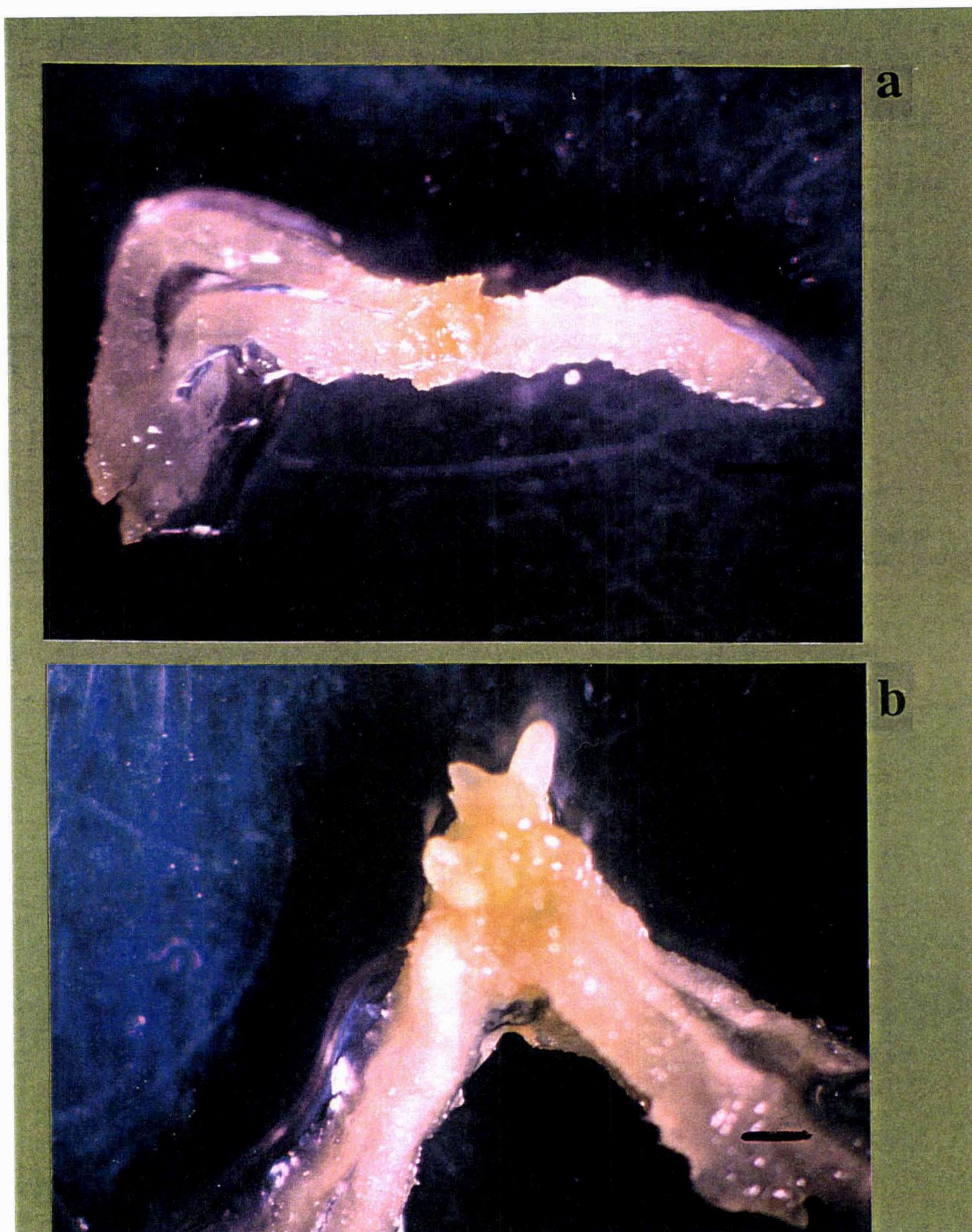


Plate 3.4 Somatic embryo aggregates

a. Clumps of three mature somatic embryos. Bar = 1.4 mm

b. Several somatic embryos fused at the shoot end. Bar = 1.1 mm

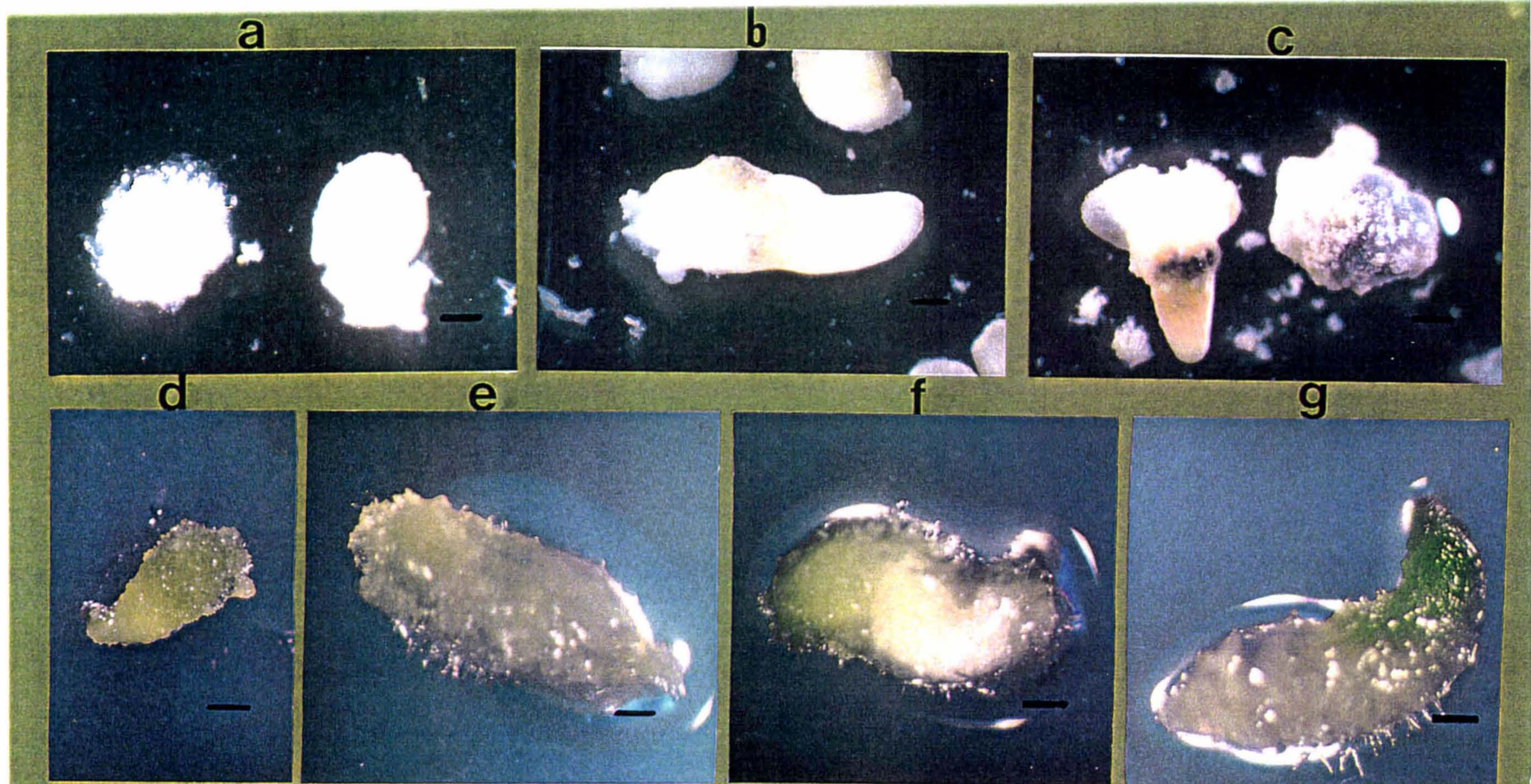


Plate 3.5a Somatic embryo development

Early stage globular embryos (a), Bar = 0.3 mm. Mature embryos (b), Bar = 0.3 mm. Globular and mature embryos (c), Bar = 0.3 mm. Mature embryo with root and shoot primordia (d), Bar = 0.2 mm. Mature embryo with root and shoot primordia (e), Bar = 0.3 mm. Mature embryo with chlorophyll pigment at shoot end (f), Bar = 0.2 mm. Early stage banana shaped mature embryo (h), Bar = 0.3 mm.

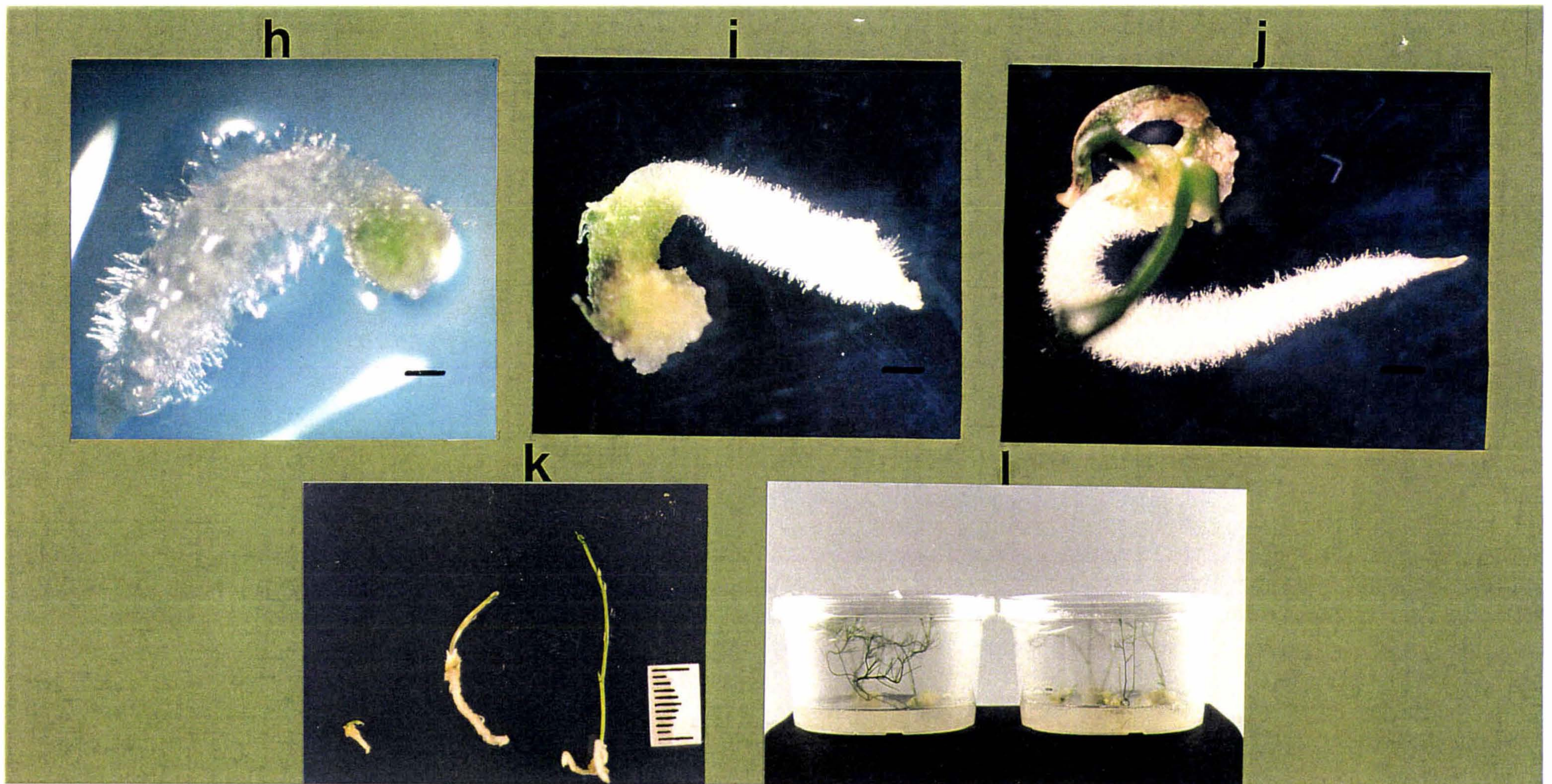


Plate 3.5b Somatic embryo development

Later stage banana shaped embryo (h), Bar = 0.3 mm. Late stage banana shaped embryo prior to emergence of shoot (i), Bar = 0.3 mm. Converted somatic embryo with developing shoot and root (j), Bar = 0.5 mm. Plantlets at different stages of development (k), Scale = 1 cm. Regenerated plantlets with developed cladophylls growing

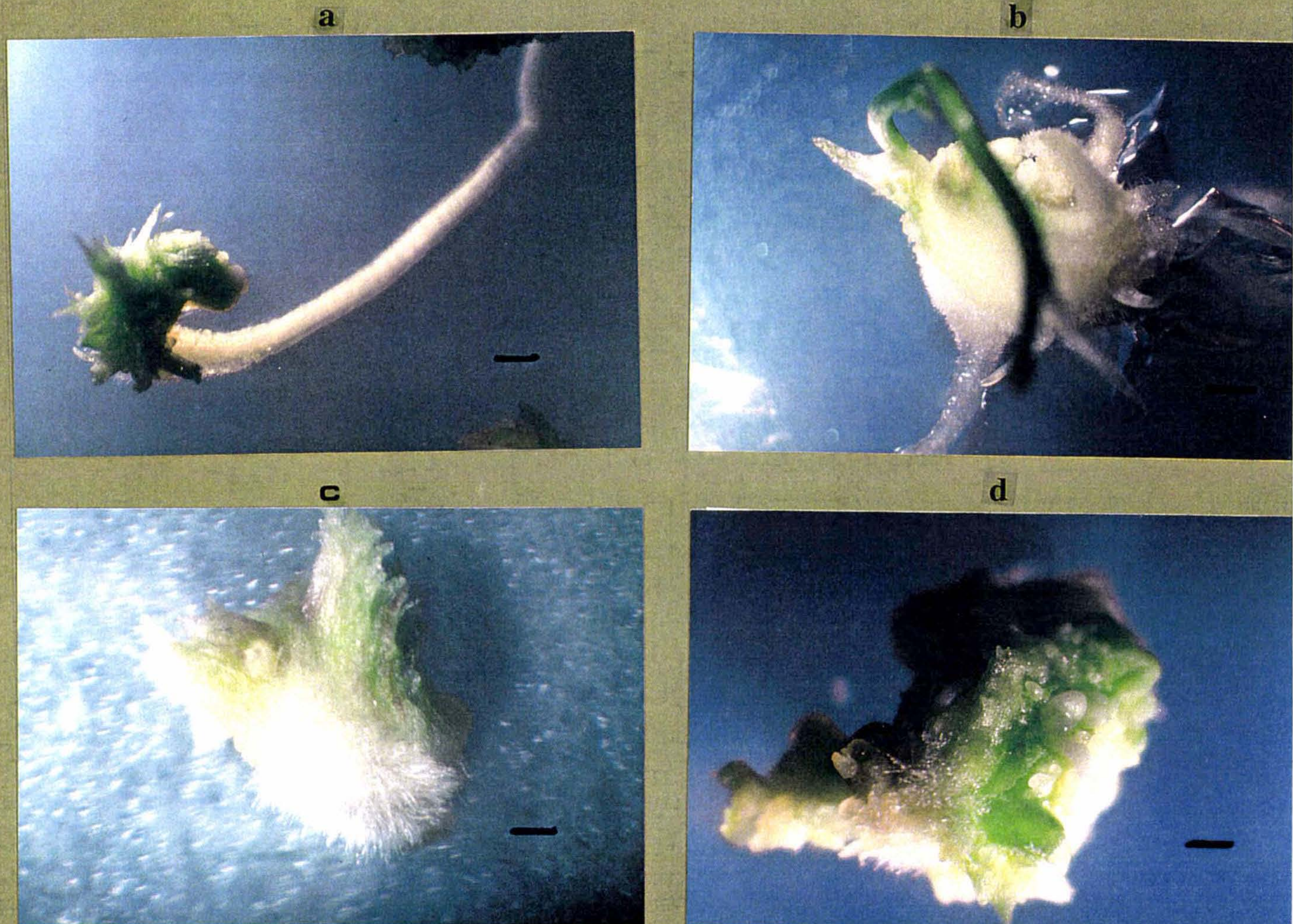


Plate 3.6 Precocious germination

a. Root development without simultaneous shoot development. Bar = 1.2 mm
b. Embryo with shoot development and abnormal root growth. Bar = 1.2 mm
c&d Abnormal development of embryo. No shoot or root evident. Bar = 1.2 mm

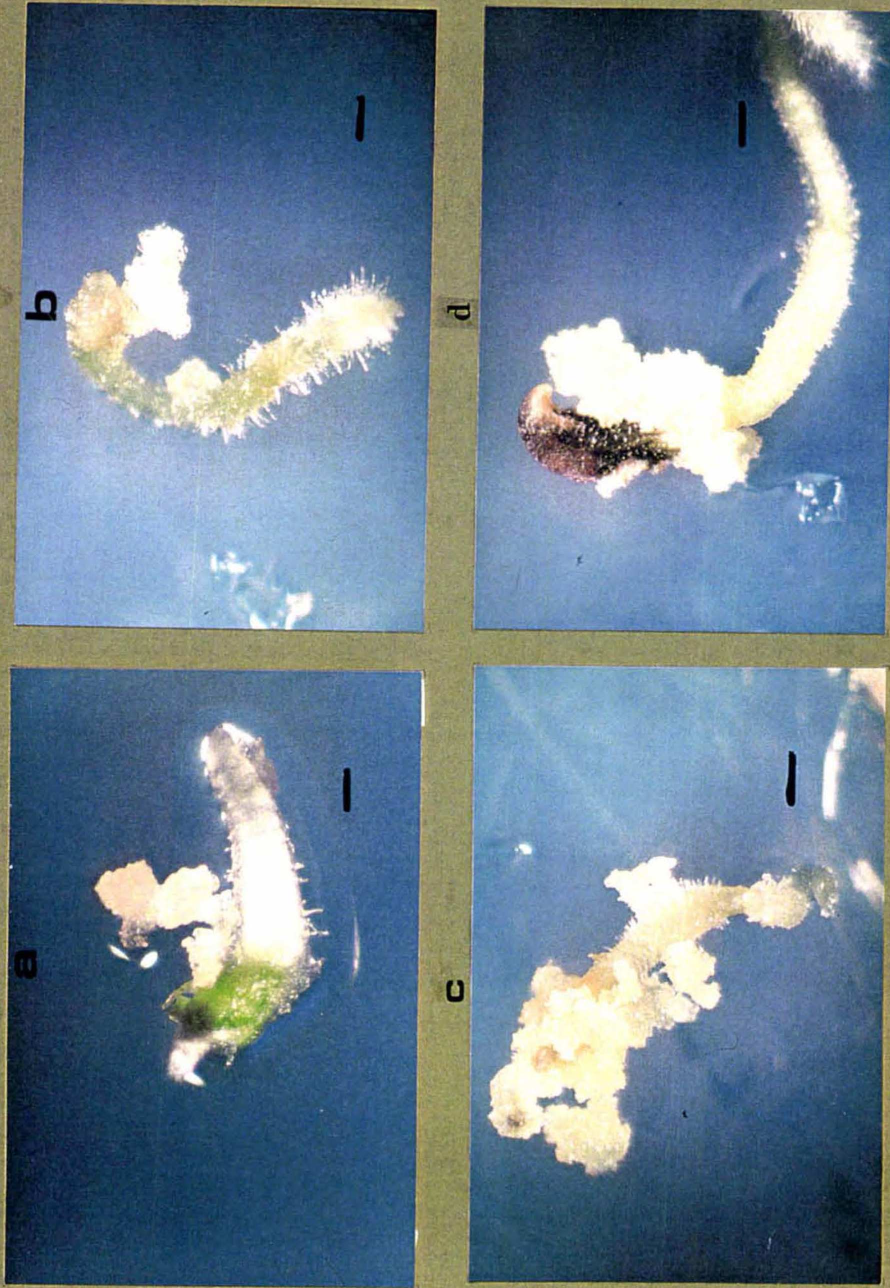


Plate 3.7 Recurrent embryogenesis

a - d Bar = 0.5 mm, 0.8 mm, 0.5 mm, and 1 mm respectively

Plate 3.8 Rhizogenesis and organogenesis

a. Rhizogenesis in suspension cultures. Bar = 5 mm
b. Rhizogenesis. Scale = 1 cm
c. Organogenesis. Cladophyll development directly from callus without root development. Bar = 0.4 mm

3.2.4 DISCUSSION

The experiments conducted, were designed to determine the most effective protocol for the development, maturation and regeneration of asparagus somatic embryos. Plantlets were recovered from somatic embryos developed using the following sequence of steps: callus induction (CI - 1.0, 3.0 and 10 μM 2,4-D), embryo induction (EI1 - MS + 6% sucrose) and regeneration (Rg4 - MS + 0.2 g/l glutamine + 3% sucrose). This protocol has the advantage in that the only step requiring the use of PGRs is the initial induction of embryogenic calli. Prolonged maintenance of plant cells on media containing PGRs increases the frequency of somaclonal variation (Geier 1991; Swartz 1991; Ghosh & Sen 1992). Therefore, any protocol that reduces the length of time cultures are grown on PGR supplemented media is desirable.

The percent conversion of embryos was variable, ranging from 11-34%. The highest percent embryo conversion was recorded for 'PC' globular embryos treated briefly at -15^{0}C. In another study, we showed that treatment of globular embryos to temperatures below 0^{0}C for 1 - 3 hrs resulted in a higher percent embryo conversion. We found chl+ embryos more responsive to cold treatment than either chl- or pur embryos. These results indicate that exposure to cold temperatures enhances embryo conversion. A detailed study using more somatic embryos of each type is required to qualify the effect, and value of low temperature treatments for embryo conversion.

Standard treatments of embryos with ABA and low osmotic potentials (Attree *et al.* 1990; Levi & Sink 1990) did not enhance embryo development beyond the globular stage in this study. Several reports have shown that ABA can be used to enhance the recovery of mature somatic embryos from a number of plant species including: *Picea abies* (Jain *et al.* 1988), black and white spruce (Attree *et al.* 1990) and papaya (Monmarson *et al.* 1995). These reports suggest that embryo response to ABA treatment may be species dependent. In addition, responsiveness to maturation treatments is also dependent on the concentration and type of ABA or osmoticum used (Attree *et al.* 1991; Dunstan *et al.*

1992; Levi & Sink 1992). Genotype differences and competence of embryos to respond to both embryo induction and maturation treatments could explain some of results obtained in this study.

Studies have shown that the onset of somatic embryogenesis requires termination and replacement of developmental gene expression with embryogenic gene expression (Merkle *et al.* 1995). This genetic reprogramming may occur as a response of somatic cells to severe stress (Dudits *et al.* 1995) such as high PGR concentrations. What is not clear however, is how to reverse embryogenic gene expression to improve embryo conversion to complete plantlets. The persistent expression of embryogenic genes may account for the high occurrence of recurrent embryogenesis observed. Maturation treatments such as ABA and low osmotic potentials are only effective on matured somatic embryos for which embryogenic gene expression has 'turned off' and developmental genes 'switched on'. Mature embryos not responding to maturation treatments may require an additional signal, e.g. a chilling stress, to terminate embryogenic gene expression. This hypothesis may explain the high frequency of conversion of globular stage embryos when treated to -15^{0}C. Merkle *et al.* (1995) reports on the use of cold treatment and desiccation treatments to facilitate embryo conversion.

3.2.5 SUMMARY

Complete plantlets were regenerated from 'PC' globular embryos maintained on PGR-free embryo induction media. Maturation and regeneration was also achieved on PGR-free media. Treatment of embryogenic calli and globular stage embryos to temperatures below 0^{0}C enhanced conversion of somatic embryos. Genotype and PGR treatment significantly influenced the number of globular stage embryos developed. Conversion of somatic embryos to complete plantlets was low (11-34 %). The physiological disorders precocious germination, recurrent embryogenesis, rhizogenesis and organogenesis were reported and contributed to a low percent of embryo regeneration. Further studies are required to determine the precise effect of maturation treatments on the conversion of globular stage embryos, and to determine the biochemical and molecular factors contributing to the responsiveness of bipolar and mature asparagus somatic embryos to maturation and regeneration treatments. These studies will allow researchers to develop more effective

and generic protocols to enhance conversion of asparagus somatic embryos for commercial production.

Acclimatisation of plantlets regenerated from asparagus somatic embryos

Abstract

The photosynthetic capacity and greenhouse survival of plantlets regenerated from asparagus cultivar Pacifica somatic embryos were investigated. Plantlets were acclimatised 3 and 9 months after transfer to somatic embryo regeneration media. Survival of plantlets acclimatised with and without storage roots and cladophylls was studied. Photosynthetic rates of 5-week-old acclimatised plantlets were compared with 5-week-old seedlings. All plantlets acclimatised without storage roots died during acclimatisation. Sixty-six percent of plantlets acclimatised with storage roots survived acclimatisation and transfer to the greenhouse environment. One hundred percent of plantlets acclimatised with storage roots, but minus cladophylls, produced new cladophylls within one week of acclimatisation. In contrast, 42% of plantlets acclimatised with storage roots, and with intact *in vitro* cladophylls, produced new cladophylls within 3 weeks, while the remaining 58% of plantlets produced new cladophylls within 5 weeks. Two to five new cladophylls were formed on all acclimatised plantlets after 5 weeks. Net photosynthesis of 5-week-old acclimatised plantlets was lower than for 5-week-old seedlings on both Days1 and 5 of transfer to the greenhouse. The photosynthetic capacity of plantlets increased significantly ($P= 0.0001$) by Day 23 of transfer to the greenhouse. Net photosynthesis increased due to the presence of new photosynthetically active cladophylls. The light compensation point for seedlings was about 8 $\mu molm^{-2}s^{-1}$ while that of acclimatised plantlets was at 40 $\mu molm^{-2}s^{-1}$. Dark respiration was 0.6 and 2.0 $\mu molCO_2.gDW^{-1}s^{-1}$ for seedlings and acclimatised plantlets, respectively. No significant difference in evapotranspiration of acclimatised plantlets and seedlings was noted. All acclimatised plantlets were phenotypically normal except for two plantlets, one with shorter internodes and the other with excessive secondary and tertiary branching on one *in vitro*-formed cladophyll. The number of *in vitro*-regenerated asparagus plantlets surviving acclimatisation can be increased by acclimatising plantlets with minicrowns that contain 2-5 storage roots, and by removal of *in vitro*-formed cladophylls prior to

acclimatisation. This study provides information to reduce the time required for efficient acclimatisation of asparagus plantlets.

Keywords

Asparagus officinalis, photosynthesis, transpiration, minicrown, storage roots, cladophylls

3.3 EXPERIMENT 3

3.3.1 INTRODUCTION

3.3.1.1 Acclimatisation of *in vitro*-produced plantlets: an overview

Successful tissue culture operations ensure the rapid multiplication of plantlets and a high percent survival when transferred out of culture (Hasegawa *et al.* 1973). Acclimatisation is the successful transfer of aseptically produced plantlets from an *in vitro* to an *ex vitro* environment. This stage is considered complete when plantlets become fully adapted to the new environment and have commenced new organ development (Sutter *et al.* 1992).

Transfer of regenerated plants from the culture vessel to the soil is often met with difficulty because *in vitro*-produced plants are not adapted to survive in the *ex vitro* environment (Pierik 1988). An increasing body of evidence indicates that the artificial environment of the *in vitro* system with its high carbon source, exogenous growth regulators, high relative humidity, and limited gaseous exchange, induces both structural and metabolic modifications in plantlets making it difficult to perform efficiently during the acclimatisation period (Dunstan 1982; Hutchinson & Zimmerman 1987; Grout 1988; Pierik 1988; Kunneman & Albers 1992; Ziv & Ariel 1992). The inability of plantlets to adapt to greenhouse conditions is associated with impaired physiological functioning of *in vitro*-formed leaves or roots which results in excessive water loss. A secondary and less important factor is the lack of a symbiotic association with microflora normally present *in vivo* (Pierik 1988). While much attention has been focused on the *in vitro* factors influencing plantlet survival during acclimatisation, factors such as day length, temperature and nutrient regime has also been known to influence plant survival during acclimatisation (Kunneman & Albers 1992).

Tissue cultured plants are typically grown *in vitro* in low light intensity and high relative humidity. The leaves of these plants do not function to their full photosynthetic

capacity and are sometimes characterised by malfunctioning stomata, reduced deposits of epicuticular material, low photosynthetic activity and deformed mesophyll (Grout 1988; Yue *et al.* 1992). *In vitro*-cultured plantlets are predominately heterotrophic and are not autotrophs immediately after removal from culture (Grout 1988). Lack of autotrophic capacity produces plantlets with no net photosynthetic gain.

Vitrification is used to describe some anatomical, physiological and morphological disorders in *in vitro*-regenerated plantlets (Ziv 1991). Although the effects of vitrification can be observed in leaves, stems, and roots, its major impact is manifested in the leaves with affected gas exchange. Vitrified plantlets can be identified by their broad, thick translucent stems and brittle leaves that are wrinkled or curled and frequently elongated (Gaspar *et al.* 1987). The relative water content of vitrified tissue is higher than normal plant tissue. Kevers *et al.* (1984) show that some vitrified plantlets have a lower dry weight (0-43%), and therefore a higher wet weight than non-vitrified plantlets.

In vitro culture conditions can also produce poorly functional or non-functional roots which can contribute to reduced survival of acclimatised plantlets (Apter *et al.* 1993a and 1993b). Roots formed *in vitro* can be non-functional for a number of reasons, including poor vascular connections between shoots and roots, poor development of root hairs and reduced root development (Pierik 1988).

Acclimatisation of tissue cultured plantlets is not only influenced by *ex vitro* environmental conditions, but also by *in vitro* culture conditions (Kunneman & Albers 1992). Therefore, any attempt to improve the rate of survival and general condition of *in vitro*-regenerated plantlets should focus on these two key phases of micropropagation. To condition plantlets for transfer from *in vitro* to *ex vitro* a number of approaches have been considered including increased light intensities (Kozai *et al.* 1988); increased light and carbon dioxide levels combined (Desjardins *et al.* 1988); reduced relative humidity or oxygen levels (Shimada *et al.* 1988; Cranes & Hughes 1990); and, the use of modified culture vessels or media (Pasqualetto *et al.* 1986; Tanaka *et al.* 1992) have also been useful. These approaches have attempted to improve the photosynthetic capacity of *in vitro*-developed leaves to increase carbohydrate reserves, reduce the

relative humidity of culture vessels in an attempt to enhance epicuticular wax deposits on leaves, and to decrease the incidence of vitrification.

3.3.1.2 Acclimatisation of *in vitro*-regenerated asparagus plantlets

Yue *et al.* (1992) observe that the photosynthetic capacity of acclimatised asparagus plantlets was lower than for seedlings or *in vitro* plantlets. For example, at a light intensity of 450 $\mu molm^{-2}s^{-1}$, net photosynthesis of seedlings and *in vitro* plantlets was approximately 0.19 $\mu molCO_2.gDW^{-1}s^{-1}$, whereas, at the same light intensity, acclimatised plantlets were at their light compensation point. This reduction in net photosynthesis is thought to be associated with high water loss from *in vitro*-formed cladophylls. Therefore, increased survival of asparagus plantlets during the acclimatisation phase should focus on these two problematic areas: low photosynthetic capacity and high water loss from *in* vitro-formed cladophylls.

To address these problems, it was shown that increasing light intensity and carbon dioxide enrichment during the *in vitro* rooting stage can significantly enhance the performance of acclimatised asparagus plantlets (Hasegawa *et al.* 1973; Desjardins *et al.* 1990; LaForge *et al.* 1991). Modification of the culture media to improve *in vitro* rooting and minicrown development improved plantlet survival (Gunawan & Sidharta 1994). With the addition of 5 µM ancymidol to the culture media an increased asparagus plantlet production was shown as well as enhanced development of storage roots and shoots (Chin 1982). Desjardins *et al.* (1987) demonstrates that decreasing the culture media osmotic potential by using high sucrose concentrations in combination with 5 µM ancymidol increased rooting of asparagus cultivars by 95%.

A report on acclimatisation of asparagus plantlets indicated the importance of *in vitro* minicrown development on plantlet survival (Conner *et al.* 1992). Acclimatisation of *in vitro*-regenerated asparagus plantlets comprising a well developed minicrown system and storage roots, improved survival of plantlets when transferred to the greenhouse. Conner & Falloon (1993) showed that high sucrose concentrations (6%) in the culture media also enhanced minicrown development and plantlet survival.

While several approaches have been used to improve survival of asparagus plantlets during acclimatisation, this stage of micropropagation still remains problematic, as is the slow growth of plantlets during and after the acclimatisation stage. Both problems limits the commercial applicability of micropropagation of asparagus clones. This experiment investigated three areas of acclimatisation of asparagus plantlets regenerated from somatic embryos: the photosynthetic capacity of plantlets, the importance of *in* vitro-developed storage roots during acclimatisation, and the effect of removal of *in vitro*-developed cladophylls prior to acclimatisation.

3.3.2 MATERIALS AND METHODS

3.3.2.1 *In vitro*-regenerated plantlets

Asparagus plantlets regenerated from 'Pacifica' somatic embryos were transferred from 90 mm petri dishes containing 10-15 ml of regeneration media (Rg4) (MS salts, 3% sucrose, 0.2g/l glutamine and 1% agar) and cultured in 300 ml plastic tissue culture tubs containing 50 ml fresh Rg4 media. Cultures were maintained at 21 ± 1°C under fluorescent tubes at 25 $\mu molm^{-2}s^{-1}$ and a 16-hr photoperiod. Plantlets were randomly selected for acclimatisation after 3 months and 9 months of culture on regeneration media.

3.2.2.2 Plantlet acclimatisation

Seven days prior to full acclimatisation, plantlets were pre-acclimatised by removing the seal from the lid of the culture vessel. In addition, 30 small holes were punched into the lid with a 1 mm size needle. Culture vessels were transferred to a growth chamber kept at 21± 1°C and light intensity of 30 $\mu molm^{-2}s^{-1}$. A 16-hr photoperiod was maintained for this pre-acclimatisation phase. After one week, pre-acclimatised plantlets were washed free of agar and transferred to 72 cell seedling trays containing a mix of peat:perlite (1:1). This growth medium was autoclaved at 1.05 kg/cm^2 for 30 min and drenched in fungicide (Benlate®) prior to transplanting. Seedling trays containing plantlets were loosely covered with clear plastic film and maintained in a growth

chamber (Contherm Scientific) kept at 21± 1°C, 95% RH, 213 $\mu molm^{-2}s^{-1}$ of light and a 16-h photoperiod. Plants were misted every 3 hrs with sterile distilled water and fertilised twice per week with a solution of Peter's professional water soluble fertiliser (M-77 chelating formula) (Scott®). After 2 weeks, the plastic film was removed and plantlets were transferred to another growth chamber and kept at a light intensity of 213 $\mu molm^{-2}s^{-1}$ for a further 3 weeks. Five weeks after acclimatisation, plantlets with actively growing shoots were selected for photosynthesis analysis.

3.3.2.3 Acclimatisation with or without *in vitro*-developed cladophylls

To assess the ability of *in vitro* plantlets to produce new shoots when acclimatised, 40 'Pacifica' plantlets cultured on Rg4 medium for 9 months, and with 2-5 storage roots were selected. Twenty plantlets were acclimatised without cladophylls (cladophylls were removed at crown level) and 20 were acclimatised with *in vitro*-formed cladophylls. For comparison, 40 plantlets without storage roots were treated similarly. All other acclimatisation procedures were as stated above. The time to the appearance of new shoots and the number of new shoots were recorded.

3.3.2.4 Seedlings

Seedlings of asparagus cultivar Syn-4 were germinated in covered glass petri dishes containing filter paper (Whatman No.1) soaked with distilled water. After 2 weeks, germinated seedlings were transferred to 72 cell seedling trays containing the same potting mix as that used for transplanting plantlets. Seedlings were maintained in the same growth chambers as acclimatised plantlets. Five-week-old seedlings were used for photosynthesis determination.

3.3.2.5 Measurement of photosynthesis and transpiration

To establish the photosynthetic response of both 5-week-old acclimatised plantlets and 5-week-old seedlings, net photosynthesis of both plant materials was obtained using different light intensities. Photosynthetic analysis was conducted at the controlled

environment (CE) facilities at the HortResearch National Climate Laboratory, Palmerston North. A modified gas exchange system used by Greer (1995) was used to collect whole-plant photosynthesis data while all data collection and measurement systems were the same as those used in Greer's study. To maintain a steady gaseous flow rate and to control CO_2 flow, dry air (BOC gases, New Zealand) was used instead of ambient air. All measurements were made at 22°C, 375 µl/l CO_2 and at a light intensity of 168 $\mu molm^{-2}s^{-1}$. Potted plantlets, with cladophylls 8-12 cm high, were placed in an assimilation chamber. The light intensity was measured at the soil surface with the lid of the assimilation chamber closed. To prevent gas exchange between soil and incoming air, the pot and potting mix were sealed with plastic bags at the crown of plantlets. As a control, photosynthesis measurements were made using pots containing only potting mix sealed in plastic bags. Plants (cladophylls and spears down to crown level) were harvested at the end of each run and the dry weight was determined. Because measurement of leaf area was impractical, photosynthetic rates were expressed on a dry weight basis. Four replicates of three plants per sample were used for photosynthesis measurements. Data were collected over a period of 5-20 min per sample and 4 readings of each replication were recorded. Photosynthesis measurements were taken both in the morning at 8:30 and in the afternoon at 3:30. Transpiration and photosynthesis data were obtained simultaneously .

3.3.2.6 Statistical analysis

While for light response curve a complete randomised block design (CRD) was used, a CRD with repeated measures analysis was used for photosynthesis and transpiration. All statistical analysis was performed using SAS Version 6.12 for Windows (SAS Institute Inc.), and treatment means were separated by LSD at the 5% significance level.

3.3.3 RESULTS

3.3.3.1 Acclimatisation of regenerated plantlets

Two hundred and thirty-nine plantlets regenerated from asparagus somatic embryos were acclimatised. Thirty-seven and 202 plantlets were acclimatised at 3 and 9 months, respectively, after embryo transfer to Rg4 media (Table 3.7). Plantlets acclimatised after 3 months growth on Rg4 media had a fibrous root system only (Plate 3.9a). An extensive root system, consisting of 2-5 thick storage roots and thinner fibrous roots, was observed on nine-month-old plantlets (Plate 3.9b and c), the exception being for plantlets regenerated from embryos derived from long-term 'PC' suspension cultures (Plate 3.9c). Storage roots are clearly shown in Plate 3.9d. Plantlets acclimatised without developed storage roots did not survive acclimatisation treatments (Table 3.8), while 66% of plantlets with storage roots survived greenhouse establishment.

3.3.3.2 Acclimatisation of plantlets without *in vitro*-developed cladophylls

The survival rate of plantlets acclimatised with and without *in vitro*-developed cladophylls was 0% and 100%, respectively, for plantlets with no storage roots and those with storage roots. All plantlets with storage roots, and with their *in vitro*-developed cladophylls removed prior to acclimatisation, produced new cladophylls within one week. Forty-two percent of plantlets acclimatised with storage roots and intact *in vitro*-formed cladophylls produced new shoots within 3 weeks, while the remaining 58% of plantlets produced new shoots within 5 weeks of acclimatisation. Between 2 to 4 new cladophylls were produced per acclimatised plantlet within a 5-week period. For some plantlets (approximately 30%), new cladophyll development commenced only after *in vitro*-developed cladophylls senesced.

3.3.3.3 Photosynthetic capacity of *in vitro*-regenerated plantlets and 5-week-old seedlings

(a) Light response curve

Net photosynthesis increased with increased light intensity for both acclimatised plantlets and seedlings (Figure 3.4a). However, net photosynthesis of cladophylls of acclimatised plantlets was significantly lower (P= 0.0001) than seedlings. The light compensation point of seedlings was 8 $\mu molm^{-2}s^{-1}$ while that for acclimatised plantlets was at 40 $\mu molm^{-2}s^{-1}$. Dark respiration was at 0.6 and 2.0 $\mu molCO_2gDW^{-1}s^{-1}$ for seedlings and acclimatised plantlets, respectively. The rate of photosynthesis increased significantly (P = 0.001) at light intensities 0, 11, and 54 $\mu molm^{-2}s^{-1}$ for seedlings and 0, 11, 88 and 149 $\mu molm^{-2}s^{-1}$ for acclimatised plantlets.

(b) Net photosynthesis and transpiration of plantlets and seedlings

Net photosynthesis for both types of plant material increased significantly (P = 0.0001) with time (Figure 3.4b). Net photosynthesis of acclimatised plantlets was significantly lower (P = 0.0001) than for seedlings at Day 1 and Day 5. No photosynthesis measurements were taken for approximately 2 weeks between Day 5 and Day 23 because acclimatised plants were too small and CO_2 flow rates could not be stabilised. No significant difference in net photosynthesis was observed between morning and evening for either plant type. There was no significant difference in transpiration of acclimatised plantlets and seedlings except for Day 5 when transpiration of seedlings was higher (P = 0.007) (Figure 3.4c). Time was the most significant main effect (P = 0.0001). Water loss for both seedlings and acclimatised plantlets was not significantly influenced by the time of day measurements were taken. No significant difference in transpiration occurred between measurements within replications. However, there were significant differences (P = 0.0001) in the transpiration of plantlets used for each replication.

3.3.3.4 Growth of *in vitro*-regenerated plantlets in the greenhouse

The appearance of acclimatised plantlets was compared with seedlings 3 months after acclimatisation. When the root systems of acclimatised plantlets and seedlings were compared (Plate 3.10), both had developed a minicrown system with storage roots. Thirty acclimatised plantlets with both *in vitro*-formed cladophylls and 3-5 *ex vitro*-formed cladophylls 10 cm or greater in length were selected and examined for phenotypic abnormalities. Twenty-eight plants had a normal phenotype, one plant had shorter internodes on new *ex vitro*-developed shoots (Plate 3.11a), while another plant had extensive secondary and tertiary branching on one *in vitro*-formed shoot (Plate 3.11b); *ex vitro*-developed shoots on this plant were phenotypically normal.

Table 3.7 Number of *in vitro*-developed 'Pacifica' plantlets acclimatised and their percent survival after 5 weeks. Treatment 1 represents 'PC' embryogenic calli initiated on 10 μM 2,4-D, treatment 2 and 3 represent long-term embryogenic calli initiated on 1.0 μM 2,4-D and 10 μM 2,4-D and treated at -15°C.

Treatment	No. regenerated plantlets	No. of plantlets acclimatised after:		Total no. of plantlets acclimatised	Fleshy storage roots	No. of plantlets surviving	% survival
		3 months	9 months				
1	169	15	154	169	+	102	60
2	41	13	28	41	-	0	0
3	29	9	20	29	+	12	41

Table 3.8 Plantlet storage root effect on acclimatisation and survival after 5 weeks growth in the greenhouse.

	No. of plantlets acclimatised	No. survived	% survival
Total plantlets acclimatised	239	114	48
Total plantlets with storage roots	174	114	66
Total plantlets without storage roots	65	0	0

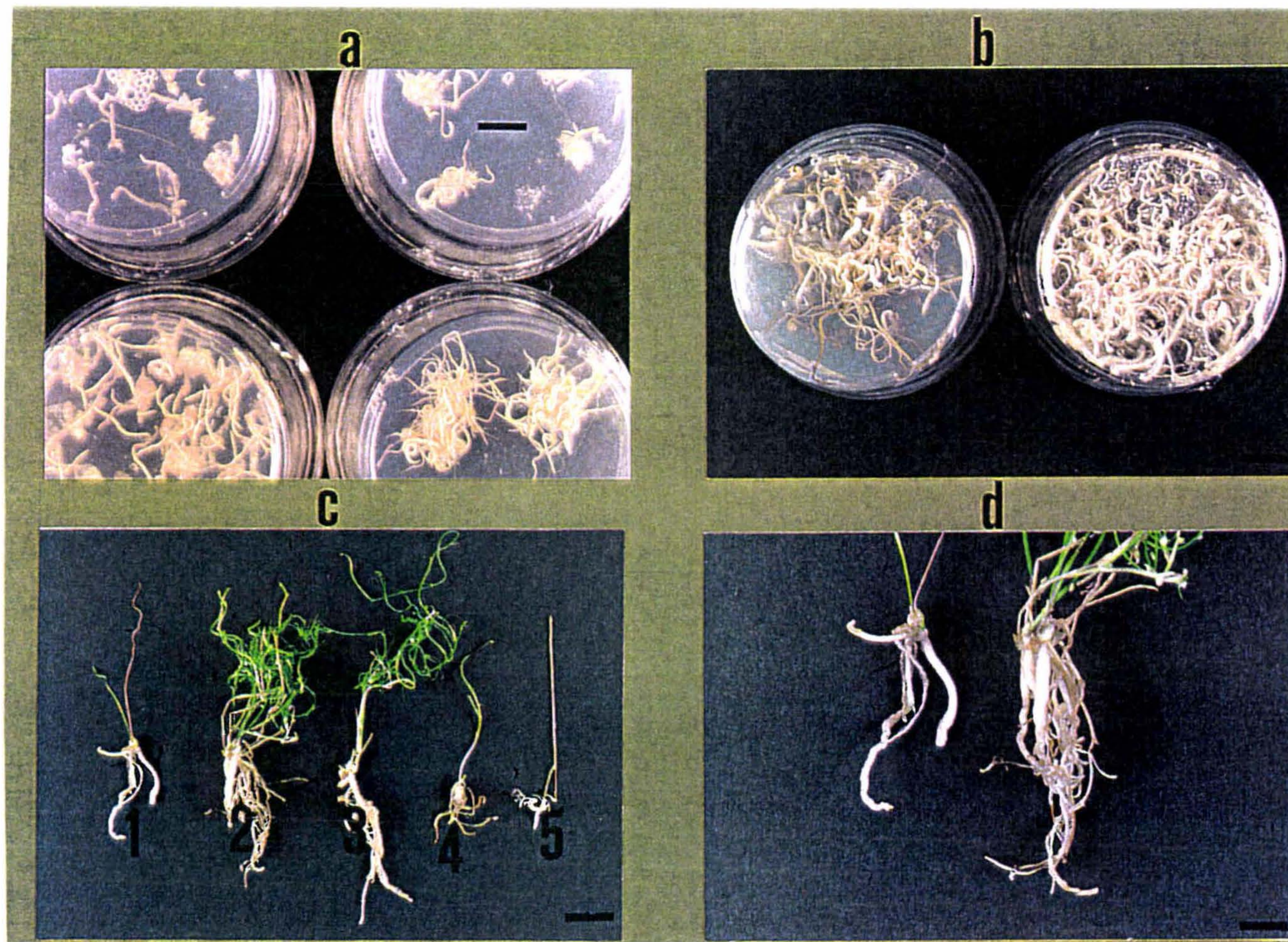


Plate 3.9 *In vitro* root development on *Asparagus officinalis* L. cultivar Pacifica plantlets regenerated from somatic embryos.

(a) Root system of plantlets 3 months after transfer to regeneration medium. Bar = 8 mm
(b) Root system of plantlets 9 months after transfer to regeneration medium. Bar = 16 mm
(c) Minicrowns with storage and fibrous root development on 9-month-old plantlets prior to acclimatisation (1-3). Fibrous root development on plantlets derived from long term embryogenic cultures (4), and root development on a 2-week-old seedling (5). Bar = 16 mm
(d) Minicrown on plantlets. Bar = 1.6 mm

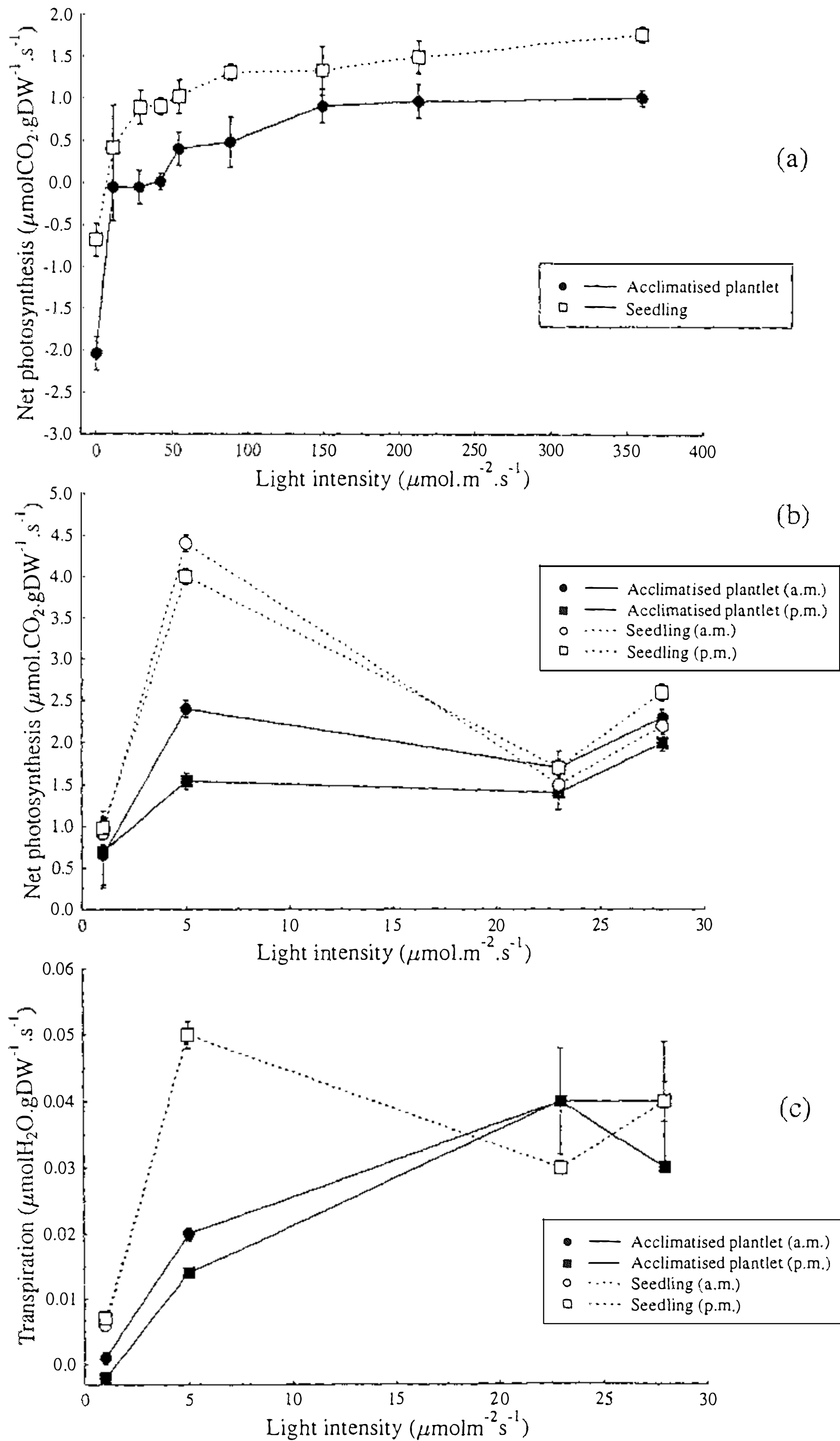


Figure 3.4 Photosynthesis and transpiration of 5-week-old acclimatised 'Pacifica' plantlets and 5-week-old 'Syn4' seedlings. Light response curve (a). Net photosynthesis (b), and transpiration (c) rates were determined at a PPF of 168 μmol$m^{-2}s^{-1}$. Measurements were made at 22°C and 375 μl/l CO_2. Vertical bars indicate SEM (n=16).

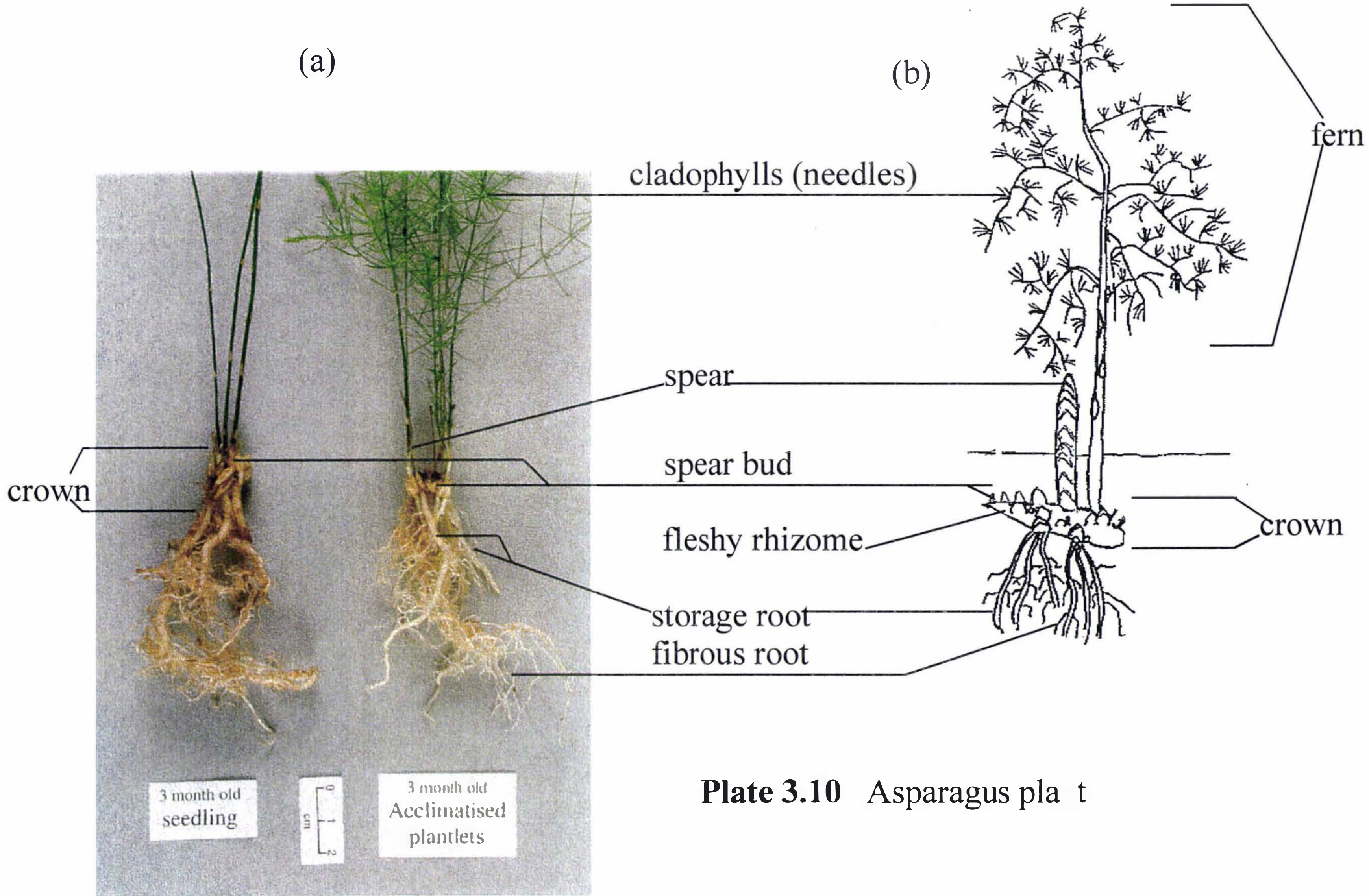


Plate 3.10 Asparagus pla t

a

b

Plate 3.11 Morphology of 3-month-old greenhouse acclimatised plantlets.

(a) Plantlet with extensive branching on cladophyll indicated by arrow head.

(b) Plantlet with short internodes indicated by arrow head.

3.3.4 DISCUSSION

Acclimatisation of plantlets regenerated from asparagus somatic embryos was enhanced significantly by *in vitro* development of a minicrown with 2 to 5 storage roots. These results were similar to those obtained by Conner *et al.* (1992) who show that development of storage roots on micropropagated asparagus plantlets not only increase survival rates of acclimatised plantlets, but also result in rapid post-acclimatisation growth in the greenhouse. In this study, minicrown development on plantlets was achieved 6 to 9 months after transfer to Rg4 media containing 3% sucrose. Conner & Falloon (1993) demonstrate that low osmotic potentials, achieved by addition of 6% sucrose to the culture media, promote minicrown development on asparagus plantlets within 6 weeks. These results suggest that after initial growth of mature somatic embryos on Rg4 medium, plantlets can be transferred to media containing 6% sucrose to enhance minicrown development. This treatment would reduce the time required for minicrown and storage root development. For commercial purposes reduced time in culture would be advantageous.

Removal of *in vitro*-formed cladophylls, from regenerated plantlets with storage roots, prior to acclimatisation also enhanced survival of plantlets when transferred to the greenhouse. Enhanced survival could be due to the removal of inadequately photosynthesising shoots that would have utilised storage reserves in the roots without replenishing reserves. Removal of these poorly photosynthesising cladophylls promoted new shoot development from spear buds. New photosynthesising shoots could provide storage reserves for new bud development more readily than pre-existing cladophylls. Removal of cladophylls would also protect acclimatised plantlets from water stress which can have a detrimental effect on survival (Sutter *et al.* 1988; Marin & Gella 1988). The current commercial practice for acclimatising asparagus plantlets is to remove *in vitro*-developed shoots to about 2 cm above the crown (Abernethy & Conner 1992). This study shows a beneficial effect from removal of *in vitro*-formed shoots down to the crown level when acclimatising plantlets.

Net photosynthesis of plantlets one day after transfer to the greenhouse environment was low (0.8 μmol CO_2g $DW^{-1}s^{-1}$). At this stage of acclimatisation, only *in vitro*-developed cladophylls were present on plantlets. These results suggest that *in vitro*-developed cladophylls did not fix CO_2 efficiently. Net photosynthesis of plantlets had increased significantly by 23 days after transfer to the greenhouse. This increase in net photosynthesis coincided with the presence of recently developed photosynthesising cladophylls. The rate of photosynthesis of these new shoots was comparable to that of seedlings.

It should be noted that 5-week-old acclimatised plantlets and 5-week-old seedlings were not at the same developmental stage and, therefore, direct comparisons of their performance should be made with caution. However, the use of 5-week-old seedlings does give a basis from which to judge the performance of acclimatised plantlets. Yue *et al.* (1992) also used 5-week-old acclimatised plantlets and 5-week-old seedlings in a similar study to the one conducted here which demonstrates that the photosynthetic capacity of acclimatised plantlets was lower than for seedlings and was insufficient to support autotrophic growth. This was evident by the 100% death of plantlets acclimatised without storage roots. Storage roots contain carbohydrate reserves able to support new cladophyll development. These results indicate that *in vitro*-developed shoots were not autotrophic and that those surviving plantlets did so by utilising energy reserves stored in storage roots. Yue *et al.* (1992) show no significant difference in net photosynthesis of acclimatised asparagus plantlets and seedlings. In addition, Yue *at al.* report that photosynthesis was sufficiently high in plantlets to support autotrophic development during acclimatisation. The differences in photosynthetic response in plantlets acclimatised by Yue *et al.* and those used in this study are difficult to explain, but may be due to dissimilar *in vitro* growth conditions and plant genotype.

No significant difference in transpiration of either acclimatised plantlets or seedlings was noted. The higher transpiration rate observed for seedlings 5 days after transfer to the greenhouse may be influenced by difficulties experienced in maintaining a steady gas flow rate on this particular day. Several reports indicate that one of the major factors influencing plantlet survival after transfer from the culture vessel is excessive water loss (Sutter *et al.* 1992; Yue *et al.* 1992). Loss of water has been associated with

partly opened stomata and a thin or absent epicuticular wax layer on the leaves of plantlets. Marin & Gella (1988) note that cuticular transpiration accounted for 95% of water loss within an hour of acclimatising *Prunus cerasus* L. The results in the present study show that after 5 weeks of acclimatisation, the evapotranspiration of acclimatised asparagus plantlets and seedling was comparable, results consistent with those of Yue *et al.* (1992) who show that transpiration from cladophylls of *in vitro*-cultured asparagus plantlets was 3.4 and 1.8 times higher than acclimatised plantlets and seedlings, respectively, when measured at 450 $\mu molm^{-2}s^{-1}$. These results suggest that the acclimatisation period is essential to prevent excessive water loss from *in* vitro-developed asparagus cladophylls.

Phenotypic abnormalities observed in acclimatised plantlets were shorter internodes and excessive secondary and tertiary branching in cladophylls. Whether these abnormalities were physiological and due to *in vitro* culture conditions or due to changes in genetic structure of plantlets will be addressed in a later section.

3.3.5 SUMMARY

The survival of asparagus plantlets in the greenhouse was enhanced by the presence a minicrown with storage roots and also by the removal of *in vitro*-developed cladophylls prior to acclimatisation. Net photosynthesis of *in vitro*-formed cladophylls was lower than for newly formed *ex vitro* cladophylls. Evapotranspiration of acclimatised plantlets and seedlings was similar. Successful acclimatisation of asparagus plantlets can be achieved by maintaining plantlets on regeneration media until 2 to 5 storage roots form, and further improved by removal of all cladophylls prior to acclimatisation. These treatments would also reduce the time required for acclimatisation and transfer of plantlets to the greenhouse compared to current commercial practices.

Further *in* vitro studies are required to develop a regeneration medium that enhances the simultaneous development of the shoot and root primordia of mature embryos, while, reducing the time required for development of minicrown and storage roots.

3.5.6 ACKNOWLEDGEMENTS

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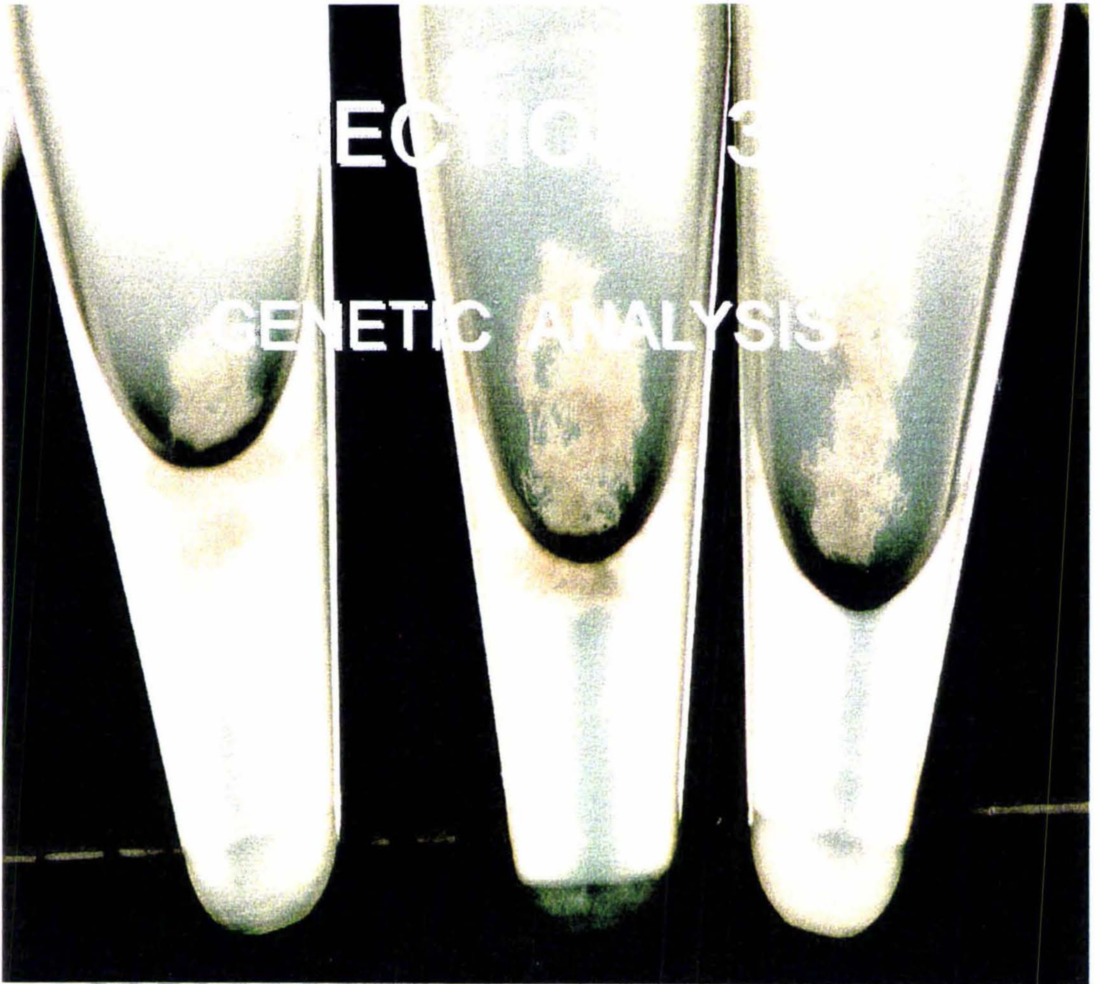
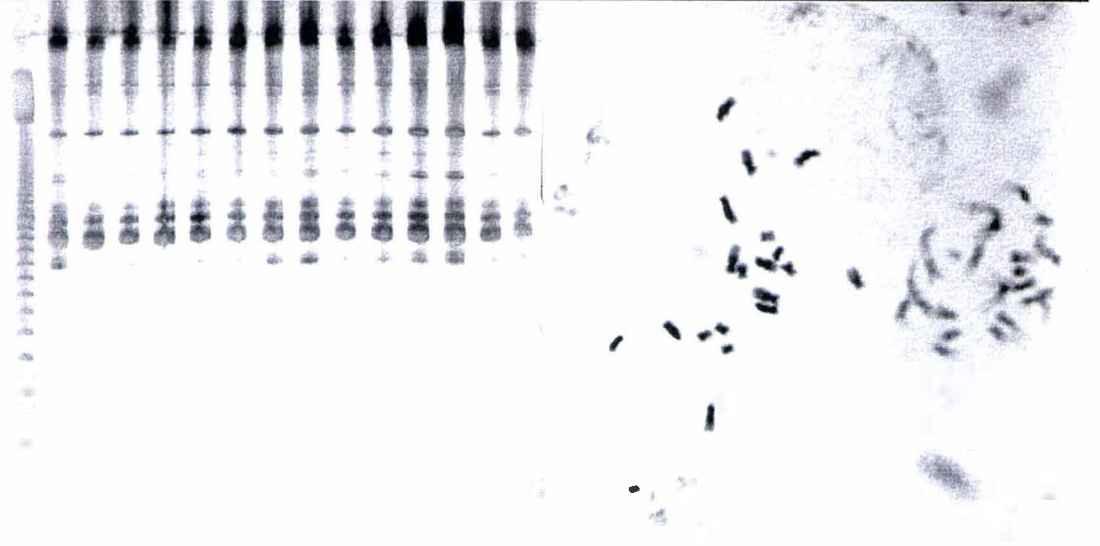
SECTION 3

GENETIC ANALYSIS





CHAPTER 4

VARIABILITY WITHIN *IN VITRO*-CULTURED PLANT CELLS

4.1 INTRODUCTION

As the occurrence of genetic variation during culture is of concern since it can result in the regeneration of 'off-type' plants (Swartz 1991), one of the primary objectives of commercial micropropagation is to mass-produce plantlets identical to the parent plant. *In vitro*-associated genetic variation has been detected in cells for morphological characteristics (color changes, dwarfism, changes in growth habit or productivity), biochemical characteristics (changes in secondary metabolite products) and chromosome number and structure (Evans & Sharp 1983; Ahuja 1987). However, genetic variation is not restricted to cells cultured *in vitro*, but is a naturally occurring phenomenon widely exploited in plant breeding and crop improvement programs (Ahuja 1987). Natural variation in organisms is achieved through genetic recombination and spontaneous mutations. Genetic stability within an organism is controlled by a range of internal mechanisms which can range from repair mechanisms to correct gene mutations, to recognition signals at every developmental stage to limit hybridization within species (Demarly 1986).

The following sections will briefly discuss naturally occurring genetic variation. As the focus of this research is on asparagus somatic embryogenesis, genetic variation observed in *in vitro* culture will be discussed in more detail.

4.2 VARIATION IN VEGETATIVELY PROPAGATED PLANTS

Natural mutations (defined as a change in chromosomes or genes of a cell affecting genomic structure and development of offspring), occur spontaneously (the frequency varying for different genotypes and type of tissue within the same genotype) and have a greater affect on cells in less organised systems such as callus and inner tissues of

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meristems, than the more stable cells of the apical meristem. Bhatia *et al.* (1985) estimated the frequency of spontaneous mutations in plants to be between 10^{-4} to 10^{-7} per locus.

4.2.1 Plant sports

Plant 'sports' are permanent deviations from the normal phenotype and their ontogeny is associated with a permanent genetic change of the apical meristem (Balkema 1971; Swartz 1991). Genetic changes can appear as altered phenotype in leaves, flowers and fruits (Dermen 1960; Wolff 1996) and are easily observed. In contrast, mutations affecting physiology or biochemistry are not easily detected if they are not expressed in the phenotype as noted in the Delicious apple (Dayton 1969) and camellias (Savige 1992).

4.2.2 Chimeras

Individual plants with more than one genetically different tissue growing separately, but adjacent to each other and arranged in layers or sectors in the stem, are classified as chimeras (Kester 1983). Chimeras normally originate from mutations occurring in a dividing cell located in any of the three histogenic layers of the meristem, and occasionally from graft-hybridisations (Hartmann *et al.* 1990). Depending on the location of the mutation one of the following chimeras are prevalent (Balkema 1971; Hartmann and Kester 1983; Marcotrigiano 1990; Swartz 1991): Periclinal chimeras, the most common and stable type, have one or more complete cell layers mutated. Mericlinal chimeras are similar to periclinal chimeras except that the mutated tissue is found only in part of one of the histogenic layers and does not extend around the shoot. This type of mutation is generally unstable. Sectorial chimeras are more common in roots than shoots and generally occur early in embryo development before distinct layers develop in the meristem. Sectorial mutations are usually unstable and different parts of the plant can develop either as normal, mutated or periclinal chimeras. Horticulturally, this instability is evident as a reversion to a form more typical of the wild type (Vaughn 1983).

4.2.3 Variegation

Plant variegation is often caused by mutant plasmagenes affecting chlorophyll development (Marcotrigiano 1990). Segregation of green and white plastids within plant cells during mitosis (Hartmann & Kester 1983) produce distinctly different colour zones in affected leaves and stems (Dermen 1960). This is characteristic of true variegation and can either be of genetic or cytological in origin. Variegated organs typically develop with a mosaic, mottled, marbled, flecked or sectored appearance (Tilney-Bassett 1991). Many economically important examples of variegation occur in citrus and ornamental monocotyledons and dicotyledons (Dermen 1960). Variegation may also occur when a plant is unhealthy or infected with a pathogen, e.g. viruses (Tilney-Bassett 1991).

4.3 CAUSES OF GENETIC CHANGE IN PLANT GENOMES

Genetic flux in plant genomes is associated with stress or traumatic events affecting an organism (McClintock 1984; Flavell 1985). McClintock (1984) demonstrates that rapid restructuring of the maize genome can occur in response to stressful factors occurring both inter- and intra-cellularly. Factors such as interspecific or intergeneric hybridisation, presence of infectious organisms, nutrient deficiency, and conditions of *in vitro* culture have modified gene action, restructured the genome and modified chromosomes (McClintock 1984; Cullis 1985). Susceptibility of plant genomes to stress appears to be restricted to some plant lines, not all species, or all members of the same species, respond similarly to identical stressful conditions (Cullis 1985).

Genetic variation can occur through sexual reproduction; sexual polyploidisation or dysfunctional sexual events (Kindiger & Dewald 1996); transposition of repeat sequences (Dellaporta & Chomet 1985); exchange of genetic material among plant organelles (Lonsdale 1985); mutations in vegetative material (Dermen 1960);

transformation at the cellular level; fusion of haploid cells (Hienz & Mee 1971); the presence of infectious organisms (Gheysen *et al.* 1985); and *in vitro* culture (Parfitt & Arulsekar 1987).

4.3.1 Reproductive mechanisms to facilitate genetic change in plant genomes

Normally, genetic diversity in plant genomes is achieved by sexual reproduction. Reduced genetic variability is expected in inbred, clonal or selfed cultivars, whereas, in out-crossing cultivars more genetic diversity is expected (Morell *et al.* 1995). By necessity, genetic variation in asexually propagated plant species, must be achieved by other mechanisms. For example, in the apomictic tetraploid Eastern gamagrass (*Tripsacum dactyloids* var. *dactyloids* (L.) L.) genetic diversity is achieved by an incomplete and dysfunctional sexual mechanism which does not require the introduction of new germplasm (Kindiger & Dewald 1996).

Genome incompatibility caused by interspecific and intergeneric hybridisation can initiate mechanisms that may result in genome restructuring and reorganisation in a hybrid plant, its progeny and may be expressed over successive generations. Modification of plant genomes as a consequence of intergeneric hybridisation promotes the formation of new species (McClintock 1984).

4.3.2 Repeat sequences

A major contributor to genetic instability in plant genomes is the presence of several copies of a series of gene families of repeat DNA sequences found dispersed throughout the genome (Flavell 1985). These hypervariable repeat sequences are categorised according to their function or their arrangement within a genome (Flavell 1985) and include minisatellites, microsatellites, variable number of tandem repeats (VNTR) (Parkin & Wetton 1991), or transposable elements (McClintock 1984; Nelson 1990). The relative abundance of each repeat family within a genome is variable (Brady *et al.* 1996) and can constitute as much as 50-90% of total genomic DNA in some species (Flavell 1985; Bachmann *et al.* 1991). Amplification, transposition or deletion

of tandem arrays can cause genetic instability within a genome by altering the number of repeats within the array (Flavell 1985). These changes may be due to duplication errors with enzymes involved in recombination and replication mistaking one repeat copy for another thereby facilitating changes in DNA structure.

4.3.3 Movement of DNA between plant organelles

Although cytoplasmic organelles contain DNA (Johansen *et al.* 1995), their interaction with each other and the mode of inheritance of their genetic material is not widely understood (Mogensen 1996). Lonsdale (1985) observed that some chloroplast and mitochondrial gene products are expressed by nuclear genes, suggesting that at some time in the development of plant genomes a transfer of genetic material from these two organelles to the nucleus could have occurred. The precise mechanism of inter-organelle transposition between chloroplast and mitochondria is not known. However, there is speculation that the close association between these two organelles may lead to their fusion and subsequent intra-molecular recombination of their genomes. Another mechanism for chloroplast-mitochondria DNA transposition may rely on the outer membrane continuity between these two organelles where passive transfer of DNA molecules may be facilitated if the membrane is permeable. Chloroplast DNA in the cytoplasm can also be transferred to the mitochondrial genome by a mechanism similar to bacterial transformation.

4.3.4 Variation caused by the presence of infectious organisms

Plant genomes are also altered by the presence of some infectious organisms, a well documented example being *Agrobacterium tumefaciens* reviewed by Gheysen *et al.* (1985). Briefly, a part of the genome, the transfer DNA (T-DNA) of this soil bacterium, is transferred, expressed and stably inherited in plant cells. The direct result of this additional foreign DNA in plant cells is crown gall tumours evident at or near the junction of the root, stem or crown of the plant.

Gene expression in some plants may be altered by the distribution of a viral organism within the plant (Swartz 1991). This normally causes modification in pigment synthesis and distribution in regions of the plant infected with the virus (McClintock 1984). For example, infection of maize plants with barley stripe mosaic virus mRNA can cause sufficient stress to activate transposable elements present in the genome.

4.3.5 Genetic engineering

Plant genomes have also been altered by the deliberate introduction of foreign DNA by plant scientists (Srivastava 1991). Genetic engineering, also referred to as genetic transformation or recombinant DNA technology, relies on the modification of plant DNA with DNA from another organism. Normally, a modified Ti plasmid from *Agrobacterium tumefaciens* is used as a vector to introduce foreign DNA into plant cells (Vasil 1987). However, in some monocotyledonous species like asparagus, *A. tumefaciens* mediated gene transfer is problematic (Potykus *et al.* 1995) and by necessity must be achieved by bolistic (particle gun) techniques or by electroporation of protoplasts (Mukhopadhyay & Desjardins 1994c; Guangyu 1996). Agronomically important crops have been genetically modified to produce transgenic plants expressing foreign DNA. These include a modified δ-endotoxin gene of *Bacillus thuringiensis* in rice (Fujimoto *et al.* 1993) and a modified movement protein gene of tomato spotted wilt virus (TSWV) in tobacco plants (Prins 1997).

4.3.6 Epigenetic effects

Stably inherited patterns of gene expression are referred to as epigenetic (Meins 1974; George & Sherrington 1984). Some phenotypic differences in plant tissue may depend on the part of the total package of genetic information within the cell that is activated. These tissue dependent factors are independent of the genetic constitution of the plant (George & Sherrington 1984). Examples of epigenetic effects are the heritable and reversible change

in PGR requirement in *in* vitro-cultured cells (Meins 1974; Reinert *et al.* 1977), and a decline in embryogenic potential of somatic cells (Reinert *et al.* 1977).

4.4 *IN VITRO*-ASSOCIATED GENETIC VARIATION

Variation detected in *in vitro* regenerated plantlets, derived from somatic cell culture, is somaclonal variation and variant clones recovered are called somaclones (Evans *et al.* 1984; Ahuja 1987). Another type of variation observed in culture is gametoclonal variation, arising from the culture of haploid gametic tissue. Both somaclonal and gametoclonal variation can result from either pre-existing genetic heterogeneity of the explant source, or from changes caused by *in vitro*-induced stress (Müller *et al.* 1990; Ogura 1990; Cecchini *et al.* 1992).

4.4.1 Somaclonal variation in micropropagated plant species

Tissue-cultured plantlets may be regenerated either directly through organ culture (bud or nodal culture) or indirectly via a callus phase (adventitious embryogenesis or bud formation) (Cassells 1985). Somaclonal variation occurs at higher frequencies in long-term callus cultures or suspension cultures derived from callus, than in the more stable organ cultures (Ahuja 1987). Experimental evidence suggests that variation in *in vitro*-cultured plantlets is a result of aberrations existing in the donor tissue, plus variation induced by culture conditions (Evans *et al.* 1984; Parfitt & Arulsekar 1987; Patel & Thorpe 1987).

Armstrong and Phillips (1988) record the frequency of variation in maize regenerants to be greater (37.2%) in friable embryogenic callus than in organogenic cultures (24.2%). However, Chowdhury and Vasil (1993) were unable to detect any DNA variation in sugarcane plantlets regenerated through an embryogenic pathway. Differences in these findings may be attributed to the genotype of the explant or to differences in culture

conditions. Normal frequencies of somaclonal variation are between 1-3% per regeneration cycle (Skirvin *et al.* 1993).

Genetic variants are reported for a range of plant characteristics including morphological, physiological and chromosomal variation (Parfitt & Arulsekar 1987; Reuther 1990b). Evaluation of initial *in vitro* regenerants (R_0) and their offspring (R_1) reveal that the following genetic changes occur during culture: changes in chromosome number and structure (Evans *et al.* 1984; Geier 1991); single gene mutations in the nucleus (Ahloowali 1986); changes in chloroplast and mitochondrial DNA (Day & Ellis 1985; Evans *et al.* 1984); and, mitotic crossing over and activation of transposable elements (Peschke *et al.* 1987; Geier 1991).

Initiation of plant tissue into *in vitro* culture imposes stress to the explant, causing restructuring of the genome in some plants that can give rise to the altered phenotypes discussed in this section. Altered phenotypes are observed in some plants regenerated from culture while others are lethal and do not survive (McClintock 1984). Somaclonal variation has been reported in a number of micropropagated plant species (Heinz & Mee 1971; Swartz 1989; Davies 1986; Geier 1988; Reuther 1990b; Isabel *et al.* 1996), and has been reviewed extensively (Chaleff 1983; Scowcroft 1985; Ahuja 1987; Karp 1989; Semal & Lepoivre 1990).

4.4.2 Factors influencing *in vitro* genetic variation

Several factors affect genetic stability of *in vitro*-cultured plantlets including the species, genotype and ploidy level; age of the plant and the type and source of explant; composition of medium and cultural conditions; length of time in culture, i.e. duration of callus phase; frequency of transfer; growth pattern; and, mode of regeneration (Ahuja 1987; Lindsey & Jones 1989; Geier 1991).

4.4.2.1 Ploidy level and genotype

The ploidy level and genotype of the starting material are important factors determining the extent of variability in culture (Geier 1991). There is evidence that ploidy levels lower than normal are unstable. For example, somaclonal variation is highest in mono-haploid, lower in di-haploid and lowest in tetra-haploid potato genotypes. In contrast, when variation is compared between polyploid and diploid genotypes, polyploid species are found to be more tolerant to chromosome variation. Likewise, evidence also shows that there is higher variability in interspecific hybrids compared with their parental species (Swartz 1991).

4.4.2.2 Explant source and age

Pre-existing genetic variation within the explant may contribute to variability in *in vitro*-developed callus and regenerated plantlets. Explant age is critical and explants, such as meristematic regions or very young tissue, with a low potential for variation, are recommended (Swartz 1991). Normally, regeneration from tissue explants results in a lower percent somaclonal variation than regeneration from protoplasts of the same initial material (George & Sherrington 1984; Geier 1991).

4.4.2.3 Culture environment

The effect of medium supplements on the genetic stability of cultured tissue are often conflicting and not easy to construe. Plant growth regulators such as auxin and cytokinins may enhance chromosome variability by disrupting mitosis (Geier 1991). For example, 2,4-D stimulates DNA synthesis and endoreplication which can lead to nuclear fragmentation (Swartz 1991), and has also been implicated where increased mitotic crossing-over occurs. Genetic changes caused by 2,4-D can occur at any time during the culture process as such changes are linked directly with normal growth and cell division. Other factors such as insufficient nucleic acid precursors, the use of some antibiotics, high

concentrations of inorganic nitrates, and the accumulation of ethylene in culture vessels have the potential to induce mutations in culture (Swartz 1991).

4.4.2.4 Length of culture period

The length of time between subcultures and the number of subcultures affects the genetic stability of *in vitro* cultures. Generally, variability increases with increased number of subcultures and time in culture (Geier 1991; Swartz 1991; Cecchini *et al.*1992). In some studies, genetic stability appeared to be unaffected by the number of subcultures (Swartz 1991). However, there is a general trend towards increased genetic variability with increasing time in culture. Kumar and Walton (1992) show that aging F_1 hybrid callus cultures of (*Elymus canadensis* x *E. trachycaulus*) increased the frequency of changes in ploidy level of regenerated plants. Variation in chromosome number was noted in 12% of regenerants. Likewise, Armstrong and Phillips (1988) observed that the frequency of chimerism in regenerants, derived from embryogenic and organogenic maize cultures, increased with increasing culture age. Using molecular techniques, Müller *et al.* (1990) demonstrate that lower levels of DNA polymorphisms could be detected in rice plantlets regenerated from callus incubated for 28 days than among those incubated for 67 days. It is not clear if the increased frequency of somaclonal variation, due to the length of the callus growth phase, is a direct effect of the maintenance of a proliferative callus phase or due to prolonged exposure to growth regulators such as 2,4-D, or due to a synergistic effect of both factors (Müller *et al.* 1990; Geier 1991; Swartz 1991).

4.4.2.5 Pattern of growth and mode of regeneration

In vitro developed plantlets regenerated via a callus phase typically have a higher percent of variability than those derived through organ cultures. Histological evidence does not support this, particularly in monocotyledons (Geier 1991). In micropropagated monocotyledons, e.g. asparagus, in addition to disorganized cell development, a type of callus frequently occurs similar to a mass of organ initials. These tissues undergo a pattern of cell division like meristems in intact plants and maintain their genetic stability when

cultured. This theory has been proposed to explain the relative stability of monocotyledonous regenerants like lily, anthurium and asparagus (Geier 1991).

Different types of callus tissue may develop from a single explant source (George & Sherrington 1984). Therefore, in addition to the 'meristematic-like' callus, other less morphogenic friable callus may develop that normally exhibit a high degree of somatic variability. Experimental evidence indicates that for some monocotyledonous species, embryogenic cultures, often derived from morphogenic callus, are more stable than organogenic cultures (Swedlund & Vasil 1985). Similarly, Chowdhury and Vasil (1993) used restriction fragment length polymorphism (RFLP) techniques to show that sugarcane mitochondrial DNA remained unchanged during somatic embryo culture. Their investigation examined plantlets regenerated from calli, cell suspensions, cryopreserved cell suspensions and protoplasts. The lack of genetic variation may either be a result of mitochondrial DNA being unaffected by *in vitro* culture conditions or the selection of the embryogenic process for the regeneration of genetically normal cells. However, Armstrong and Phillips (1988) show that for some species, for example maize, this degree of genetic stability is not the case, finding friable, embryogenic maize cultures to be naturally more cytologically unstable than organogenic cultures.

4.5 DETECTING GENETIC VARIABILITY IN REGENERANTS

In vitro-induced somatic variation is not always easily observed in regenerants (Lassner & Orton 1983). For example, changes in gene expression or base pair sequence may not result in any easily detected phenotypic variation. Therefore, it is critical that techniques employed to determine genetic stability of cultured cells not only examine morphological differences in regenerants, but also any changes that may occur at the molecular level.

Researchers may use a range of techniques to estimate the genetic uniformity of plantlets developed *in vitro*. These techniques can be subdivided into two main categories involving phenotypic and genetic approaches (Lindsey & Jones 1989). In the following sections some of the more common techniques as well as some of the more recent approaches used to observe *in vitro*-induced variability will be discussed.

4.5.1 Phenotypic analysis

4.5.1.1 Morphological characteristics

Morphological characteristics, such as growth habit, fruit and leaf morphology are easy to observe and score (Lindsey & Jones 1989). These traits are observed late in the developmental phase of the crop under investigation. For commercial micropropagation it would be desirable to determine the percent genetic variation at an early stage of development. For this reason, more detailed and sensitive analysis is required. These analytical techniques can be utilised within the system as quality assurance and control measures. While some variation is inevitable, these checks can determine the level of variation that does occur. It is then left to researchers, commercial micro-propagators and growers to determine the level of variation acceptable for each crop. Macroscopic, microscopic and image analysis techniques are used during morphological analysis of *in vitro*-cultured tissue and regenerated plantlets. These techniques are dependent on the phenotypic characteristics of regenerated plantlets (Geier 1991).

4.5.1.2 Biochemical analysis

Biochemical analysis of cultured cells and regenerated plantlets is often based on the evaluation of specific proteins or other endogenous compounds that are produced through expression of specific genes under developmental regulation. Cytochemistry may be effectively used to determine the difference between cells which may appear to be genotypically uniform (Patel & Thorpe 1987). Common cytochemical techniques involve, for example, localization of RNA, proteins, carbohydrates, lipids and specific enzyme activity.

4.5.1.3 Protein electrophoretic analysis

Electrophoretic techniques can detect proteins produced by specific gene expression (Montembault *et al.* 1983; Tedesco *et al.* 1991). These techniques are limited as they only

detect variation in gene expression for one unique product, while other variation is undetected. Despite these limitations, techniques such as isoenzyme analysis and total protein profiles are often utilised to detect genetic polymorphism in plant genomes.

Isoenzyme analysis has been used for the study of genetic variation inherited in plant genomes, and the physiological and developmental differences occurring in *in vitro* cultures (Heinz & Mee 1971; Parfitt & Arulsekar 1987). Isoenzyme systems can be used for direct detection and measurement of genetic variation in cultured cells (Eastman *et al.* 1991); visualization of the loss of chromosomes in somatic hybrid tissue; the provision of experimental systems for the study of somatic recombination; karyotypic changes; point mutations and transposable elements (Lassner & Orton 1983); and, to develop heterozygous isozyme gene markers to identify double-haploids and hybrids (González-Castañón & Carbajal-Carcedo 1996). In asparagus, isoenzyme gene markers have been used for the establishment of linkage groups which can be used for breeding purposes, to map sex chromosomes and for the identification of sex-linked markers which can allow plant sex to be determined non-destructively (Maestri *et al.* 1991; Brettin & Sink 1992).

4.5.1.4 Secondary product analysis

Secondary product analysis has not been used to identify somaclonal variants in *in vitro*-produced plantlets due to the specific but limited nature of the analysis (Bretting & Widrlechner 1995; Morell *et al.* 1995).

4.5.2 Genetic analysis

4.5.2.1 Cytological analysis

Cytological analysis is commonly used to detect *in vitro*-induced variation by determining if the chromosome number of the donor plant has been maintained throughout culture. Chromosome morphological changes are normally studied at the metaphase stage of cell division as this is when the chromosomes are easily observed (Parfitt & Arulsekar 1987).

At this stage, centromeres and their positions on chromosomes, secondary constrictions and satellites can be readily observed (Parfitt & Arulsekar 1987; Lindsey & Jones 1989).

Although cytological analyses of *in vitro* cultures and plantlets can be utilised to show the genetic variation, they are, however, not effective enough to detect subtle changes at the molecular level which do not appear as altered chromosome number or structure. Therefore, attention must be focused on genome analysis at the DNA level (Lindsey & Jones 1989).

4.5.2.2 Molecular analysis

Molecular DNA techniques are some of the most powerful tools used by biologists to further advance their understanding of the genetic composition and evolutionary history of a wide range of organisms. Molecular DNA markers were first developed to study animal genomes; however, over the past decade plant scientists have adapted these techniques for a variety of purposes (Beckman & Osborn 1992; Nybom 1994). Molecular markers are dependent on variation in DNA sequence among and within genomes to reveal polymorphisms. They have been used extensively in genomic mapping exercises, in the classification and identification of genomes, in the cloning of specific loci of interest, for the protection of breeders' rights, to determine genetic similarity/relatedness among and within species, and for the detection of somaclonal mutants (Munthali *et al.* 1992; Beckman & Osborn 1992).

The advantages of using molecular markers to detect somaclonal variation, over morphological or biochemical markers, are:

- many molecular markers are phenotypically neutral, thereby allowing direct observations of the plant genotype, unlike morphological and biochemical markers which allow observations of the phenotype (Matsumoto & Fukui 1996);
- identification can be made at the whole plant, tissue or cellular level;
- the co-dominant behaviour of alleles at most molecular marker loci;

- the presence of three different genomes (nuclear, chloroplast and mitochondrial) which may evolve independently of each other (Novy *et al.* 1994; Link *et al.* 1995);
- molecular markers can be utilised for analysis of any fraction of the genome whether or not it codes for a functional gene product (Engels 1981); therefore, all mutational events can potentially be detected (Clegg 1990); and,
- molecular markers, as well as biochemical markers like isoenzyme markers, can be scored at the seedling stage (Paterson *et al.* 1991).

However, extensive use of molecular markers may be limited by the number of polymorphic markers for which assay procedures are available, by the facilities and resources required for such analysis (Paterson *et al.* 1991; Stuber 1992), and by the high cost involved in performing analysis (Clegg 1990).

4.5.3 Molecular techniques useful for detecting somaclonal variation

Some of the more common techniques used in plant cultivar identification and to detect genetic changes in *in vitro* cultures will be discussed in this section. Further details for some of the protocols used in molecular analysis can be obtained from Sambrook *et al.* (1989a) and Hewitt *et al.* (1991).

4.5.3.1 Restriction Fragment Length Polymorphism (RFLP)

When genomic DNA is cleaved using restriction endonucleases (enzymes that recognise a specific nucleotide sequence of typically 4-6 nucleotides), DNA fragments of different lengths unique to that genome are produced (Gillet 1991). Any variation in the size of allelic DNA fragments is attributed to a loss or gain of a restriction site which may result from point mutations, deletions, insertions translocations or inversions (Lavi *et al.* 1994). After digestion of genomic DNA with restriction enzymes, and fractionation of genomic digests by gel elctrophoresis, RFLPs are detected either as discrete bands on

agarose gels or by hybridising genomic DNA fragments to a probe (Sharma & Sharma 1994). If there is any variation in the restriction sites in the genome, then a difference in the restriction banding pattern is observed (Lindsey & Jones 1989; Gillet 1991).

Use of RFLP analysis to detect genetic variation in a particular genotype has some advantages including: an observed higher level of allelic variation when compared to either isoenzyme or morphological markers; mendelian segregation; co-dominance of markers; and, the results of the analysis, which is performed at the molecular level, are independent of developmental regulation or epigenetic effects (Restivo *et al.* 1995). Extensive use of RFLP analysis is limited by the length of time to complete one analysis procedure and the use of radioactive DNA probes (Rafalski *et al.* 1993; Nybom 1994), and the difficulty in automating the system through all stages (Rafalski *et al.* 1993). Some non-radioactive detection systems have been developed for use in RFLP analysis, thereby limiting the need for radioactive probes. Application of RFLP technology in cultivar identification may also be restricted by the limited number of alleles at a given loci (Rongwen *et al.* 1995).

To-date RFLP markers have been used in the development of preliminary linkage maps for higher plant species, including *Asparagus officinalis* L. (Restivo *et al.* 1995; Caporali *et al.* 1996) and cassava (Angel *et al.* 1993).

4.5.3.2 Polymerase chain reaction (PCR)-based DNA fingerprinting techniques

Polymerase chain reaction techniques are used to amplify specific DNA fragments within a genome or to screen a genome for polymorphism (Foster *et al.* 1993; Morell 1993). For this reason, PCR is a powerful tool used to identify and characterise plant genomes (Towner & Cockayne 1993; Morell *et al.* 1995). Polymerase chain reaction-based markers have some advantage over RFLP markers, for example, PCR procedures are simpler and less labourious. In addition, PCR markers can reveal more information about a genome than RFLP and biochemical markers. As a consequence, PCR markers

are widely used to analyse plant genomes, in molecular cloning, for cultivar identification and detection of somaclonal variants.

Several implementations of the polymerase chain reaction are useful for detecting somaclonal variation in plant material grown *in vitro*, these will be outlined in the following sections.

(a) Random amplified polymorphic DNA (RAPD) analysis

Analysis using RAPDs has many applications and is used extensively to develop molecular markers useful to detect somaclonal variation. The technique allows short arbitrarily chosen DNA primers, of known sequence, to amplify those regions of the genome where the primer has bound. The amplified products generated are unique and dependent on the DNA target as well as the sequence of the primer (Lee *et al.* 1993). Polymorphisms within and between cultivars are generated when nucleotide bases between primer binding sites are changed. These changes may be due to single base substitutions in primer binding sites around the DNA sequence, deletions of a primer site, insertions that may either change the size of a DNA segment without affecting its ability to be amplified, or due to inversions (Williams *et al.* 1990).

The use of RAPD techniques (also known as Arbitrarily Primed PCR - AP-PCR) to fingerprint genomes was first demonstrated independently by Welsh and McClelland (1990) and Williams *et al.* (1990). Random amplified polymorphic DNA techniques have the advantage that they are fast and require small amounts of DNA for analysis and are amenable to automation (Rafalski *et al.* 1993; Saméc & Našinec 1996). Unlike RFLP and other PCR-based techniques, RAPD analysis does not depend on prior knowledge of the molecular structure of the genome being investigated (Welsh & McClelland 1990).

A limitation of RAPD analysis is related to the dominance of RAPD markers (Lavi *et al.* 1994; Bretting & Widrlechner 1995). In addition, poor reproducibility of RAPD profiles between different laboratories have been cited as another limitation of the technique.

However, the high sensitivity of RAPD assays may be considered an asset in detecting somaclonal variation in culture.

Applications of RAPD analysis include detection of genetic variability in chrysanthemum (Wolff & van Rijn 1993; Wolff *et al.* 1993); determination of the origin of interspecific lilac hybrids (Marsolais *et al.* 1993); identification of ornamental plants, for example, the rose (Torres *et al.* 1993; Matsumoto & Fukui 1996) asparagus (Khandka *et al.* 1996); and calla cultivars (Hamada & Haqimori 1996); and for the detection of somaclonal variation in sugarcane (Taylor *et al.* 1995); asparagus (Dan & Stephens 1997) and white spruce (Isabel *et al.* 1995) cultivars produced during *in vitro* culture.

(b) Amplified fragment length polymorphism (AFLP) analysis

Amplified Fragment Length Polymorphisms (AFLPs) is another PCR-based technique used to analyse plant genomes based on the selective amplification of subsets of restriction fragments from total genomic DNA digests (Vos *et al.* 1995). The technique involves restriction of total genomic DNA with a combination of two restriction endonucleases, ligation of oligonucleotide adapters to the restricted DNA fragments to generate a DNA template for amplification; pre-selective PCR amplification using primers that recognise the complex of adapter and restriction site followed by selective amplification with primers that amplify a subset of preselected fragments, and after amplification, reaction products are separated on gel such as acrylamide (Vos *et al.* 1995) or Separide® (Lockhart & McLenachan 1997) and analysed.

AFLP technology can be used to fingerprint plant genomes (Vos *et al.* 1995), and to identify and isolate polymorphic regions that can be used in analysis of plant genomes (Lockhart & McLenachan 1997). The use of AFLPs is in its infancy for cultivar identification and its use for detecting somaclonal variation or assessing the genetic stability of plantlets regenerated through any micropropagation technique has not been reported.

(c) Other PCR-based techniques

Other PCR-based techniques such as DAF (DNA amplification fingerprinting) analysis (Caetano-Anollés *et al.* 1995; Prakash *et al.* 1996), microsatellite and related techniques (Wolff *et al.* 1995; Brady *et al.* 1996) have proven useful for cultivar identification and, therefore, can be used to detect somaclonal variation occurring during *in vitro* culture of plants.

4.6 SUMMARY OF OBJECTIVES FOR SECTION 3

Somaclonal variation in *in vitro* cultures and regenerated plantlets has been highlighted as a major problem limiting the commercial applicability of micropropagation in some plant. The following chapters of this section evaluated the genetic stability of the asparagus genome throughout somatic embryogenic culture. Further details of the objectives and experimental approach for this section were outlined in Chapter 1.

These objectives were achieved by analysing long-term embryogenic calli maintained on medium containing PGRs for 1½ years, long-term embryogenic cells maintained as suspensions on PGR-free media for 1 year, and regenerated plantlets. Analysis was made using RAPDs and cytological techniques. The potential use of extra-cellular proteins to reveal genetic variation was also investigated using protein gel electrophoresis techniques. The merits and limitations of these techniques have been outlined in previous sections of this chapter.

CHAPTER FIVE

Detection of variation among and within asparagus hybrids using random amplified DNA (RAPD) markers

Abstract

The reliability of random amplified polymorphic DNA (RAPD) techniques to amplify polymorphisms in the asparagus (*Asparagus officinalis* L.) genome was investigated. DNA fragments generated by 10-base primers were separated on 1.5% agarose or 8% polyacrylamide gels, and the sensitivity of ethidium bromide and silver staining of amplified DNA products analysed on these gels was compared. Resolution of DNA bands on polyacrylamide gels was superior to that on agarose gels. Silver staining was more sensitive than ethidium bromide staining. The gel type used to separate DNA bands, and the staining technique used influenced the number of bands visualised for each DNA profile generated. The six asparagus cultivars used were distinguished by unique banding patterns generated by each primer, OPC-12, for example, generating polymorphic markers unique to three of the cultivars investigated, ASP (500, 400, and 300 bp), TU (700 bp), and (PC 550 bp). Clear differences were noted in DNA profiles generated for samples of seed established cultivars: SN_7 (1300 bp); SN_13 (1900, 1800, 1700, 1100, and 1000 bp); UC_5 (1800, 1500, and 1400 bp); UC_6 (2000, 1050, and 600 bp); and UC_11 (1600 bp). The investigation indicates that RAPD markers can be used to characterise asparagus cultivars, and that the technique is sensitive enough to reveal differences within seed-raised commercial cultivars. RAPD technology has the potential to detect somaclonal variation occurring during micropropagation.

Keywords

Asparagus officinalis, micropropagation, primers, RAPDs, ethidium bromide, silver stain, agarose, polyacrylamide

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A manuscript based on this experiment has been accepted for publication by New Zealand Journal for Crop and Horticultural Science.

5.1 EXPERIMENT 4

5.1.1 INTRODUCTION

Commercial micropropagation of asparagus clones requires regenerated plantlets to be genetically uniform. For this reason, it is necessary to establish reliable techniques to determine their genetic stability as the effect of genetic variation on yield within a clone has yet to be established. If *in vitro* techniques promote genetic variation, it is important to qualify some of the factors contributing to variation, and to quantify the extent of this variation. In the past, attention has been focused on the use of morphological, biochemical, and cytological techniques to determine the frequency of somatic mutations occurring during micropropagation (Heinz & Mee 1971; Patel & Thorpe 1987; González-Castañón & Carbojal-Carcedo 1996). However, both morphological and biochemical techniques are influenced by epigenetic effects and, as with cytological analysis, often fail to detect subtle genetic changes occurring at the molecular level that could have a significant impact on crop quality and yield. This is particularly important for perennial crops like asparagus that may be productive for 10-20 years.

Recently, molecular techniques such as restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNAs (RAPDs) have been used to characterise genomic DNA (Welsh & McClelland 1990; Williams *et al.* 1990; Xiao & Christie 1994), and to detect somatic mutants in some plant species (Kaemmer e*t al.* 1992; Deng *et al.* 1995). Analytical molecular techniques provide a methodology that allows evaluation of the plant genome independent of environmental factors, physiological state, or developmental regulation. RAPD techniques have the added advantage over RFLPs of being relatively simple and quick to perform requiring only small quantities of DNA for analysis. In addition, no prior knowledge of genomic DNA sequence is required for RAPD analysis (Williams *et al.* 1990). As a consequence, RAPDs have been used to identify and characterise closely related plant species and cultivars (Munthali *et al.* 1992; Torres *et al.* 1993; Yang & Quiros 1993).

Before RAPD techniques can be used either for characterisation of closely related asparagus cultivars or to detect somaclonal variation occurring during *in vitro*

production of asparagus clones, the repeatability and sensitivity of the RAPD approach must be evaluated. This report investigated: the reliability of RAPD DNA fingerprints generated by selected 10-base primers; the ability of RAPD techniques to distinguish among asparagus cultivars; and, the sensitivity of the technique for revealing differences within a seed-raised commercial cultivar.

5.1.2 MATERIALS AND METHODS

5.1.2.1 Plant material

Three of the asparagus cultivars Pacifica (PC), UC157 (UC), and Syn 4 (SN) used were maintained at the greenhouse units at Massey University. Both 'UC' and 'SN' were established from seed, whereas 'PC' was established from clonal material. Cultivars Turoa (TU), Aspiring (ASP) and Karapiro (KP) were clonal material obtained and maintained as tissue-cultured liners from Aspara Pacific Ltd, Christchurch, New Zealand. Plant material used in this investigation is listed in Table 5.1.

5.1.2.2 DNA extraction

Before DNA extraction, samples of actively growing young fern or spears were dried for a minimum of one week in sealed plastic bags containing silica gel. The procedure for DNA extraction was a modification of Doyle & Doyle's (1990) cetyltrimethylammonium bromide (CTAB) method and the minipreparation technique of Stewart & Via (1993) (P Lockhart pers. comm. 1996). The isolation buffer (2% CTAB (BDH), 1.5 *M* NaCl, 1% polyvinylpyrolidone 44 000 (PVP-44) (BDH), 20 m*M* EDTA, 100 m*M* Tris-HCl (pH 8.0)) was preheated to 60^0C. Milligram amounts (20-50 mg) of dried plant tissue were ground in a 1.5 ml eppendorf tube in liquid air. Hot isolation buffer (0.7 ml) was added and the sample incubated at 60^0C for 30-60 min. An equal volume of chloroform was added and the sample centrifuged briefly at 10 000 rpm. The aqueous phase was transferred to a clean tube and two thirds volume of isopropanol added. The white DNA precipitate was recovered by spooling, washed three or four times in 80% ethanol, dried, and resuspended in 50 μl sterile water

(Barnstead® nanopure). The quantity of DNA extracted was estimated by electrophoresis and separation of the sample DNA and known concentrations of lambda DNA (Pharmacia Biotech) on a 0.8% agarose gel, and comparing the intensity of bands visualised after staining with ethidium bromide (Sambrook *et al.* 1989b).

Table 5.1 Asparagus (*Asparagus officinalis* L.) cultivars evaluated by random amplified polymorphic DNA analysis.

Cultivar	Sample i.d	Sex	Propagation method
Aspiring	ASP	Male	clone
Karapiro	KP	Male	clone
Turoa	TU	Male	clone
Pacifica	PC_9	Female	clone
Pacifica	PC_12	Female	clone
Pacifica	PC_16	Female	clone
Syn 4	SN_7	Female	seed
Syn 4	SN_13	Female	seed
UC157	UC_5	Female	seed
UC157	UC_6	Female	seed
UC157	UC_11	Female	seed

5.1.2.3 RAPD procedure

Primers used for RAPD analysis were obtained from Operon 10-mer Kit C (Operon Technologies). A modification of the procedure of Williams *et al.* (1990) was used. RAPD reactions were performed in a total volume of 25 µl containing: 0.5 U *Taq* polymerase (Gibco-BRL), 2 m*M* $MgCl_2$ and 1X PCR buffer (supplied with Taq polymerase), 5 pmole primer, 100 µ*M* of DNA polymerisation mix (Pharmacia Biotech) (containing dATP, dCTP, dGTP, and dTTP), and 25 ng template DNA. The reaction mixture was overlaid with one drop (50 µl) of mineral oil. For control reactions, template DNA was excluded from the reaction mixture. To avoid contamination of DNA from other organisms, all PCR reactions were prepared in a laminar flow cabinet. Polymerase chain reaction (PCR) cycling conditions were as follows: 1 cycle of 5 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C and a final time delay cycle of 5 min at 72°C followed by an optional soak period at 4°C. This soak period was required for PCR reactions run overnight. PCR reactions were performed in a Perkin Elmer Cetus DNA Thermal Cycler 480. Amplified products were separated by

electrophoresis in a 1.5% agarose gel. A Tris-borate EDTA (TBE) buffer system was used. To avoid over-heating of electrophoresis buffer, gels were run at 4°C.

5.1.2.4 Improved resolution of amplified bands

For increased resolution, amplified products were separated on an 8% polyacrylamide gel and stained with ethidium bromide. Ethidium bromide stained gels were destained in water for 20-30 min as recommended by Andrews (1986), and then silver stained. The silver staining procedure was a modification of Igloi's (1983) modified Sammons' procedure. The modifications consisted of shorter incubation times at each step (H F Neilson pers. comm. 1996). Gels were initially washed twice for 5 min in ethanol and acetic acid, stained in $AgNO_3$ for 15 min, washed in running distilled water for 1 min, developed for 10 min, and fixed for 5 min. The procedure was completed in 36 min instead of the 190 min recommended by Igloi (1983).

5.1.2.5 RAPD analysis of 'Syn4' and 'UC157' seedlings

The DNA profiles of 100 seedlings of cultivar Syn4 and UC157 were analysed to determine the degree of intra-cultivar variability within cultivars. DNA extraction and amplification protocols were the same as used previously.

5.1.3 RESULTS

5.1.3.1 Screening of explants with 10-base primers

Initial screening of explants from each cultivar was performed with twenty 10-base primers. Analysis was based on the visual resolution of DNA bands on agarose gels. Criteria for selection were based on the ability of each primer to produce 5-10 clearly discernible bands for evaluation and comparison, as well as being able to produce unique fingerprints for several of the cultivars under investigation. Based on these criteria, the following 10 primers were selected from Operon Kit C: OPC-01, -02, -07, -08, -09, -10, -11, -12, -13, and -14. The number of amplified bands produced by each

primer, for all cultivars, ranged from 5 to 15, with an average of 11 bands per primer. Amplified DNA fragments were in the range of 400-2400 bp. The DNA profiles generated for each cultivar by 20 primers are found in Appendix Table 5.0.

5.1.3.2 Polymorphisms produced by different primers

The DNA fingerprints produced by 10 primers for cultivars PC_9 and SN_7 are shown in Plate 5.1 each primer investigated producing a distinct pattern of amplified bands. The difference in fingerprints generated for each cultivar is also evident in Plate 5.1.

5.1.3.3 Improved resolution of amplified products

Separation of amplified products was better on 8% polyacrylamide gel (Plate 5.2a) than on 1.5% agarose gel (Plate 5.2b). Staining with ethidium bromide did not reveal additional more intensely stained bands than in agarose. However, very high molecular weight bands (greater than the largest band on the marker), and very low molecular weight bands of 100 bp were evident on acrylamide gels. These bands were not visible on similar stained agarose gels (Plate 5.2b).

The gel in Plate 5.2a was destained and then silver stained. The increased sensitivity of this staining technique is clearly shown in Plate 5.2c. The intensity of the silver stained bands, and the number of bands evident, was greater than when stained with ethidium bromide. An average of 23 amplified bands were generated by OPC-12 when silver stained, more than twice the average number of bands revealed on agarose and acrylamide gels stained with ethidium bromide. Thirteen heavily stained bands were revealed on this gel, and these bands could be useful markers for further investigations. The results with OPC-12 were typical of other primers used in this study (data not shown).

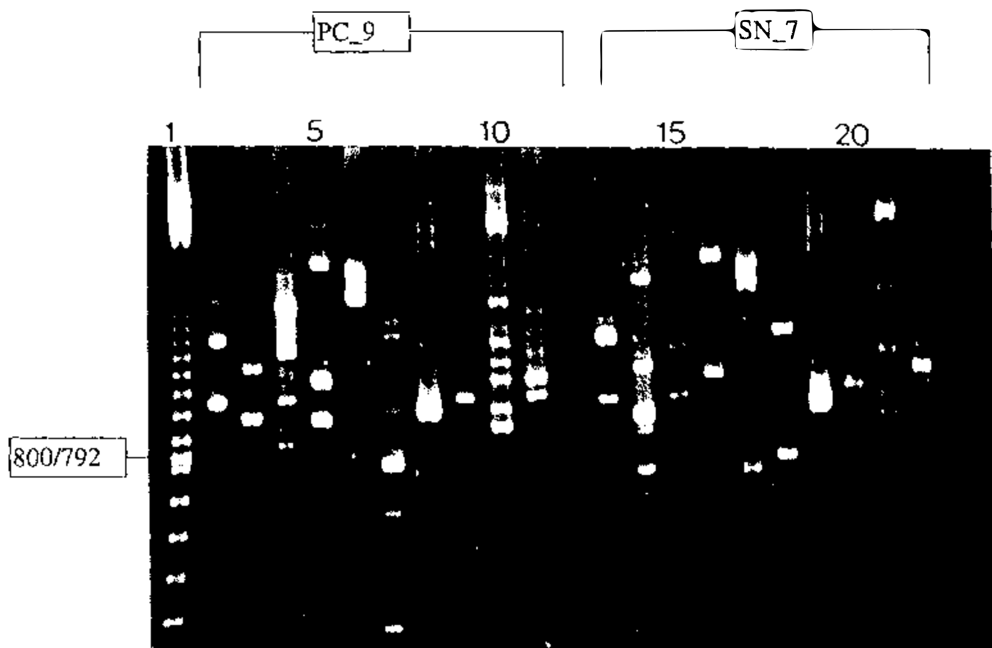

Plate 5.1 Unique random amplified polymorphic DNA profiles for PC_9 and SN_7 generated by 10 different primers. Amplified products separated on a 1.5% agarose gel and stained with ethidium bromide. 100 bp marker (Lane 1); PC_9 (Lane 2-11), DNA amplified with the following primers: OPC -01, -02, -07, -08, -09, -10, -11, -12, -13, and -14 respectively. Control (lane 12); SN_7 (Lanes 13-22) primers and order of loading onto gel was the same as for PC_9.

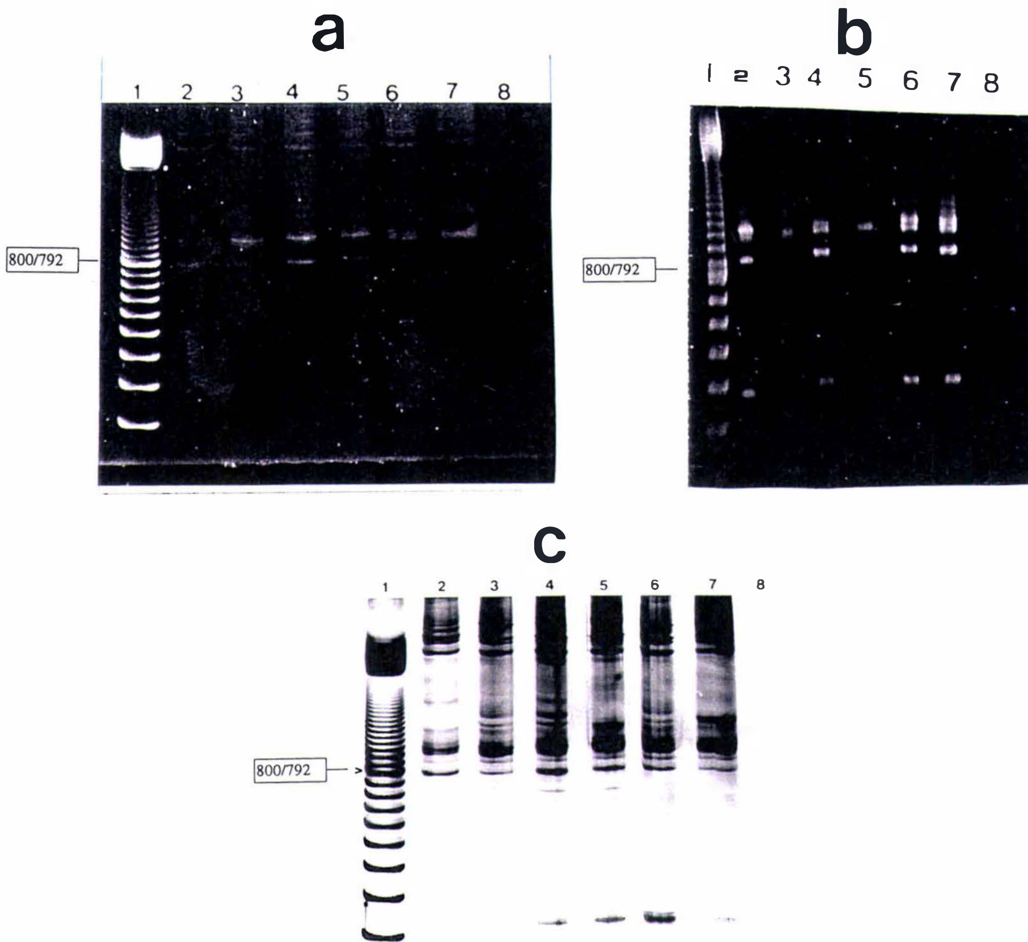

Plate 5.2 Comparison of the relative sensitivity of (**a**) ethidium bromide, and (**b**) silver staining of DNA bands for six asparagus (*Asparagus officinalis* L.) cultivars generated by OPC-12 and resolved on 8% polyacrylamide gel. Lanes 1-8 contained: 100 bp marker, ASP, KP, TU, PC_12, SN_13, UC_5, and control respectively. (**c**) DNA profiles generated for six asparagus cultivars generated by OPC_12 and resolved on agarose gel. Lanes 1-8 contained: 100 bp marker, ASP, KP, TU, PC_12, SN_13, UC_5, and control respectively.

5.1.3.4 Reproducibility of DNA fingerprints

The stability of amplified polymorphisms produced by primers was assessed for 10 independent DNA extractions of the same cultivar and 10 separate PCR reactions with bands visualised on 8% acrylamide gel. The banding patterns generated by OPC-12 for cultivar PC are shown in Plate 5.3. Similar consistencies in DNA banding profiles were obtained for the other primers screened (data not shown). Major DNA bands generated by OPC-12 were produced consistently for each separate DNA extraction and PCR reaction. The intensity of faint bands varied for some PCR reactions (Lane 5, Plate 5.3), however, the DNA profile for intense bands was similar to those produced for the other nine reactions.

5.1.3.5 Cultivar comparison and variation within cultivars

The six asparagus cultivars investigated could be distinguished by unique banding patterns produced by each primer. The differences in DNA fingerprints among asparagus cultivars, generated by all primers, were most evident on silver stained acrylamide gels. A summary of the different banding profiles generated for each cultivar by OPC-12 is shown in Table 5.2. The following polymorphic bands were amplified for only one cultivar and could, therefore, be used for cultivar identification: cultivar ASP (500, 400 and 300 bp); TU (700 bp), and PC (550 bp). Two polymorphic bands, 1400 and 800 bp, were amplified for all six cultivars, all other polymorphic bands were shared by two or more cultivars. The DNA fingerprint patterns generated for individual plants of closely propagated cultivars maintained in tissue culture (ASP, KP, TU) and plants grown in the greenhouse from tissue culture stock ('PC') were consistent. When two 'SN' and three 'UC' seedlings were analysed, the primers used did not allow discrimination among individual plant DNA profiles when analysed on agarose gels stained with ethidium bromide. However, variation among seedlings of the same cultivar was revealed when amplified products were analysed on a polyacrylamide gel stained with silver (Table 5.2). Analysis of DNA profiles of cultivar SN generated

by OPC-12 revealed that SN_7 could be distinguished from SN_13 by a 1300 bp polymorphic band, whereas SN_13 could be distinguished by 1900, 1800, 1700, 1100, and 1000 bp polymorphic bands. The following polymorphic bands distinguished among 'UC' seedlings: UC_5 (1800, 1500, and 1400 bp); UC_6 (2000, 1050, and 600 bp)); and UC_11 (1600 bp). All other amplified DNA bands were shared by two or more seedlings. RAPD analysis of an additional 100 'Syn4' seedlings using primer OPC-07 revealed 1 seedling with polymorphic bands. The DNA profiles generated by OPC-12 were identical for all seedlings. RAPD analysis of a further 100 'UC157' seedlings with OPC-07 and OPC-12 revealed 3% of seedlings with different DNA profiles. One percent and two percent of polymorphic DNA bands of 'UC157' seedlings were generated by OPC-07 and OPC-12, respectively.

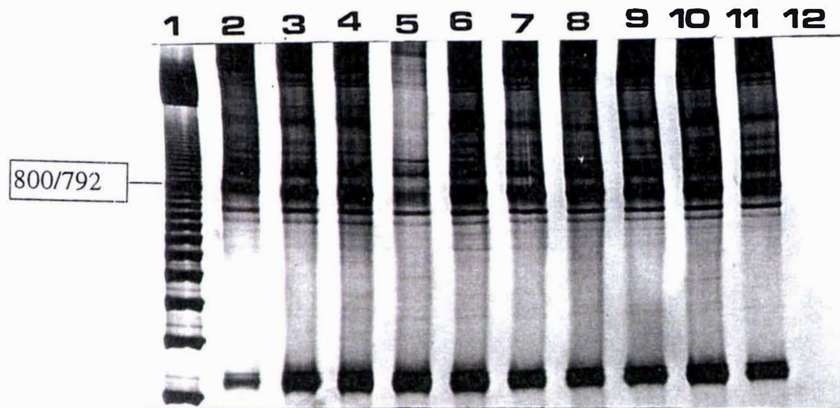

Plate 5.3 Consistency of DNA fingerprints from asparagus (*Asparagus officinalis* L.) cultivar Pacifica generated using primer OPC-12. Results of 10 separate DNA extractions and PCR reactions are shown. Amplified products were separated on an 8% polyacrylamide gel and silver stained. 100 bp marker (Pharmacia Biotech) (Lane 1); samples 1-10 (Lanes 2-11); control with no template DNA (Lane 12).

Table 5.2 Polymorphic bands present for each asparagus (*Asparagus officinalis* L.) cultivar, scores are based on profiles generated by OPC-12, separated on acrylamide gel and silver stained. Key to table symbols: v = faint bands amplified for some PCR reactions; + = presence of intense bands amplified for all reactions but which vary in intensity; ++ = very strong bands present in all reactions with no variation in intensity. 0 = absence of a band. PC* representative banding profile for all individual plants of cultivar Pacifica.

Polymorphic bands (bp)	Cultivars								
	ASP	KP	TU	PC*	SN_7	SN_13	UC_5	UC_6	UC_11
2000	+	0	v	+	0	0	0	v	0
1900	+	0	+	0	0	+	0	0	0
1800	0	+	+	+	0	+	+	0	0
1700	0	+	+	0	0	+	0	0	+
1600	0	+	+	+	v	+	+	0	0
1500	0	+	++	+	v	+	++	0	0
1400	+	+	+	+	+	+	+	+	+
1300	0	+	+	0	+	0	0	0	0
1200	+	+	+	++	+	+	+	0	+
1100	++	++	++	++	0	++	+	v	v
1050	++	0	0	0	0	0	0	v	0
1000	0	++	++	++	0	++	++	++	++
900	+	+	+	+	+	++	+	0	+
800	++	+	++	++	+	++	+	++	++
700	0	0	+	0	0	0	0	0	0
600	0	0	v	+	0	0	0	+	0
550	0	0	0	v	0	0	0	0	0
500	v	0	0	0	0	0	0	0	0
400	v	0	0	0	0	0	0	0	0
300	v	0	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0	v	v
100	0	0	0	0	0	0	0	0	0

5.1.4 DISCUSSION

Unique DNA profiles were generated for six asparagus cultivars using RAPDs. The profiles generated by each primer differed for each of the cultivars tested, demonstrating the sensitivity of RAPD techniques for differentiating among asparagus cultivars. RAPD makers were also successfully used by Khandka *et al.* (1996) to identify five asparagus cultivars and to establish their phylogenetic relationship. Major DNA banding patterns generated for the four tissue-cultured cultivars (ASP, KP, TU, and PC) were reproduced consistently over several different PCR reactions and from different DNA samples. Clear differences, noted in the profiles generated for the different samples of seed established SN and UC cultivars, are expected as both cultivar Syn4 and UC157 were produced from select vegetatively propagated parents that were not pure inbred lines. Therefore F_1 progeny are expected to be heterogeneous. RAPD analysis of 100 seedlings of cultivar Syn4 and UC157 detected polymorphic bands in 1% and 3% respectively of seedlings. Some polymorphic bands generated for each cultivar were variable and were not detected for each PCR reaction. As a consequence, although it was interesting to note their presence or absense, these bands could not be used to reliably identify asparagus cultivars.

Resolution of amplified products was greater on acrylamide gels than on agarose gels. Comparison of gels stained with silver nitrate indicates that this staining technique is much more sensitive than ethidium bromide staining. These results are consistent with those of other investigators who have shown silver staining to be 3-100 times more sensitive than other staining techniques including ethidium bromide (Beidler *et al.* 1982; Berry & Samuel 1982; Igloi 1983; Andrews 1986). Differences in mobility of DNA bands in both agarose and acrylamide gels make it difficult to directly compare DNA profiles generated on agarose and polyacrylamide gels.

Repeated PCR reactions of the same DNA samples, and separate DNA isolates, generated reproducible amplified products with each primer investigated for all cultivars. The reliability of amplified DNA profiles generated by RAPD techniques

have been questioned in the past (Munthali *et al.* 1992; Levi *et al.* 1993). Factors such as the quantity and quality of sample DNA, reaction parameters and the type of thermal cycler used have influenced the profile of the DNA bands generated (Munthali *et al.* 1992; Gogorcena & Parfitt 1994). While variation in results may be obtained between laboratories, consistent results can be maintained within a particular laboratory as long as the reaction parameters remain the same for all reactions. The results of this study indicate that the DNA profile visualised on a gel is influenced by the medium used to resolve DNA bands, and also by the band staining technique.

5.1.5 SUMMARY

RAPD technology was shown to be a useful tool to distinguish among asparagus cultivars and to reveal variation among individual seedlings established from the same cultivar. The DNA profiles generated by the primers were consistently reproduced over several PCR reactions. Resolution of amplified DNA bands by polyacrylamide gel and visualisation of bands by silver staining was more sensitive than agarose gel resolution and ethidium bromide staining. Collectively, these results indicate that RAPD analysis may be useful to investigate the occurrence of somaclonal variation occurring during plant micropropagation. RAPD markers identified in this investigation can be used to assist breeding programs by establishing phylogenetic relationships among asparagus cultivars and also to establish linkage maps for characters like sex linked genes.

5.1.6 ACKNOWLEDGMENTS

An appreciation is extended to Dr G Ionas and Mr H Neilson for useful comments.

Variation within asparagus embryogenic calli, suspension cells and plantlets regenerated from somatic embryos detected using RAPD markers

Abstract

The efficiency of random amplified polymorphic DNA (RAPD) techniques to amplify polymorphisms occurring during culture of asparagus (*Asparagus officinalis* L.) embryogenic calli, embryogenic suspension cells and plantlets regenerated from somatic embryos was investigated. The three clonal cultivars Aspiring, Karapiro and Pacifica were distinguished by unique banding patterns generated by each primer. The RAPD technique also detected changes in genomic DNA structure induced during culture of embryogenic cells. No change in genomic structure of 50 plantlets regenerated from somatic embryos was detected. Primers OPC-01, -02, -07 and -08 revealed more genomic variation than the other primers (OPC-09, -10, -11, -12, -13 and -14). These results suggest that primers OPC-01,-02, -07 and -08 may amplify genetically unstable hypervariable regions within the genome. More genetically variant DNA profiles were detected for embryogenic calli maintained on PGR medium than for suspension cells grown on PGR-free medium. Changes to DNA profiles of each cultivar were noted as an additional band usually in the lower molecular weight range (50-500bp). However, in four cases variation was noted as an absence of a band. Plant growth regulator concentration influenced the number of variant DNA profiles revealed by each primer. Embryogenic calli maintained on medium containing 2,4-D, a total of 24, 22, 9 and 6 variant DNA profiles were generated by all primers for 10, 3.0, 1.0 and 0.3 μM 2,4-D, respectively. For each plant growth regulator treatment the greatest number of variant DNA profiles were generated for cultivar Pacifica. The length of time embryogenic calli or embryogenic cell suspensions, of each cultivar, were maintained on their respective media did not appear to influence the number of variant DNA profiles revealed by primers. This study showed that RAPD technology can be used to detect somatic variation occurring during long-term culture of asparagus embryogenic cells. The research also shows that long-term maintenance of asparagus embryogenic cells on PGR-free medium is more preferable than maintenance on PGR

An abbreviated report on this experiment was presented at the IX International Asparagus Symposium, July 15-17, 1997 and was accepted for publication in the conference proceedings and will appear in Acta Horticulturae.

medium, as the incidence of variation is lower for cells grown on medium without PGRs. It was also demonstrated that plantlets regenerated through somatic embyrogenesis are genetically stable. These results suggest that variation observed during culture of embryogenic cells is not reflected in regenerated plantlets.

Keywords

Asparagus officinalis, micropropagation, primers, RAPDs, embryogenic calli, somatic variation, plant growth regulators, plantlets, regeneration

5.2 EXPERIMENT 5

5.2.1 INTRODUCTION

The use of random amplified polymorphic DNA (RAPD) techniques to detect somaclonal variation occurring during micropropagation (Hoepfner *et al.* 1993; Taylor *et al.* 1995) has not yet been explored extensively. These techniques have been used to reveal previously undetected somaclonal variants in micropropagated sugarcane cultivars (Taylor *et al.* 1995); white spruce (Isabel *et al.* 1995); both diploid and hexaploid wheat cultivars (Brown *et al.* 1993); and, chrysanthemum (Wolff & Van Rijn 1993; Wolff *et al.* 1993). RAPD techniques have also been used to develop genetic markers to identify and classify inter-specific hybrids of lilac (Marsolais *et al.* 1993) and somatic hybrids of potato (Baird *et al.* 1992; Rokka *et al.* 1994; Rasmussen & Rasmussen 1995), and to establish plant variety rights for newly developed fruit cultivars (Deng *et al.* 1993; Krahl *et al.* 1993; Novy *et al.* 1994; Stiles *et al.* 1993).

In an earlier study (Chapter 5, experiment 4) the repeatability and sensitivity of the RAPD approach to reveal both inter- and intra-cultivar differences in six asparagus cultivars was evaluated. In this study the use of RAPD techniques to reveal changes in genome structure occurring in asparagus embryogenic calli maintained on medium containing plant growth regulators (PGR) for one year, and to evaluate the genetic stability of plantlets regenerated from somatic embryos is reported.

5.2.2 MATERIALS AND METHODS

5.2.2.1 Plant material and culture conditions

Explant material: Asparagus cultivars used in this investigation were established from clonal material. Cultivars Aspiring (ASP) and Karapiro (KP) were obtained and maintained as tissue-cultured liners from Aspara Pacific Ltd, Christchurch, while cultivar Pacifica (PC) was obtained from stocks maintained in the greenhouses at Massey University. Before DNA extraction, samples of actively growing young fern or

spears from explants were collected and kept on basal callus induction medium (MS + 3% sucrose) for 3 days to ensure that samples were free of fungus or bacteria. Samples were then dried on silica gel for a minimum of one week in sealed plastic bags.

Embryogenic calli: Calli samples were obtained from the previous somatic embryo induction experiment (Chapter 3, experiment 1). Samples for DNA extraction were taken at each subculture. Calli were dried in 1.5 ml microfuge tubes stored in a container of silica gel for 2 weeks.

Embryogenic suspension cells: Embryogenic cell suspensions were collected during the somatic embryo development study (Chapter 3, experiment 2). Samples for DNA extraction were taken at each subculture. Embryogenic cells were dried in 1.5 ml microfuge tubes stored in a container of silica gel for 2 weeks.

Regenerated plantlets: Fifty Pacifica plantlets regenerated from somatic embryos, derived from callus initiated on embryo induction medium supplemented with 10 μM 2,4-D, were used. Ten of these plantlets were regenerated from somatic embryos derived from calli treated at -15^{0}C. Plantlets were acclimatised and grown in the greenhouse for 3 months. Samples of actively growing young fern or spears from plantlets were collected and kept on basal callus induction medium (MS + 3% sucrose) for 3 days and then dried for a minimum of 1 week in sealed plastic bags containing silica gel.

5.2.2.2 DNA extraction

The protocol for DNA extraction was the same as that used during the previous study (Chapter 5, experiment 4), except for the following modifications. The white DNA precipitate was recovered by spooling, washed three or four times in 80% ethanol, dried and resuspended in 50μl sterile water (Barnstead nanopure). The quantity of DNA extracted was determined by using a DyNA Quant 200 Fluorometer (Pharmacia Biotech). Calf Thymus DNA (Pharmacia Biotech) was used as a standard and Hoechst 33258 fluorescent dye (Pharmacia Biotech) used to stain DNA.

5.2.2.3 RAPD procedure

The RAPD procedure used in this study was the same as used in Chapter 5, experiment 4.

5.2.2.4 Gel electrophoresis and staining

Gel electrophoresis and silver staining protocols were the same as used in Chapter 5, experiment 4, except for the following modification. After development and fixation, gels were further incubated in a reaction termination mix (50g/l Tris, 20ml/l acetic acid) for 15 minutes, then photographed and stored in ziplock bags containing water.

5.2.2.5 Nomenclature

Each band amplified was identified by its related primer and its size in base pairs (bp). For example, OPC01-2000 refers to the 2000 bp band amplified by OPC-01.

5.2.3 RESULTS

5.2.3.1 DNA yields

Yields of explant and plantlet DNA ranged between 100 to 800 ng/µl per sample, while yields of DNA from calli and suspension cells ranged between 50 to 500 ng/µl per sample.

5.2.3.2 RAPD analysis of explants

Individual primers generated distinct DNA profiles for each cultivar, a summary of amplification products generated by each primer shown in Table 5.3. The reliability of each amplified band over several PCR reactions varied depending on their intensity. Three levels of band intensity were observed: (1) faint DNA bands were revealed for

some PCR reactions, (2) intense bands were present for all PCR reactions but with varying intensity, and (3) strong bands were reliably amplified for all PCR reactions with the same intensity. Some amplified bands were common for all cultivars, others, however, were observed for only one cultivar and could be used for cultivar identification. Cultivar-specific markers generated by each primer are listed in Table 5.4. The number of useful cultivar-specific markers generated was influenced by the type of primer and the cultivar. For example, OPC-01 generated 8, 4 and 6 distinct markers for 'ASP', 'KP' and 'PC', respectively, OPC-12 did not generate any unique markers for cultivar ASP and only 1 and 2 distinct markers for 'KP' and 'PC', respectively (Table 5.4). OPC-13 did not easily generate amplified products for all cultivars. Only after several PCR reactions were amplified products detected for cultivars ASP and PC, no amplified products were detected for cultivar KP using OPC-13.

5.2.3.3 Effectiveness of primers to reveal polymorphisms:

RAPD analysis of embryogenic calli: Primers OPC -01, -02 and -07 revealed more changes in the DNA structure of calli of all cultivars than any of the other primers. More polymorphic markers were generated for cultivars ASP and PC than for cultivar KP (Table 5.5). The total number of variant DNA profiles revealed by all primers, for each cultivar, was as follows: PC (47), ASP (20) and KP (3). Most of these variant DNA profiles were generated by OPC-01. Primer OPC-09 did not reveal changes in genomic DNA of the three cultivars.

RAPD analysis of embryogenic suspensions: Variant DNA profiles were detected for samples of each cultivar maintained as embryogenic cell suspensions on PGR-free medium. Polymorphic markers useful in identifying samples with altered genomic structure is shown in Table 5.6. Four, three and one primer (s) revealed differences in DNA structure of cultivar ASP, KP and PC, respectively. Most of the changes in DNA profiles, of both embryogenic calli and suspension cells, were noted for low molecular weight bands between 50 and 500 bp.

RAPD analysis of regenerated plantlets: The DNA profiles of 'Pacifica' plantlets regenerated from somatic embryos were identical to that of the original explant. No changes in genome structure of planlets was revealed by any of the 10 primers used. An example of the genetic stability of DNA profiles of 'Pacifica' plantlets generated using OPC-08 is shown in Plate 5.4.

5.2.3.4 Specific polymorphisms revealed for each cultivar:

RAPD analysis of embryogenic calli: Several differences in DNA profile of each cultivar were revealed by some of the primers used in this study. A summary of the primers and their associated markers useful for revealing variation in DNA profiles of samples of embryogenic calli is shown in Table 5.7. Normally, variation was observed as additional bands not present in the DNA profile of the original explant. However, in 4 instances ASP (S8) 0.3 μM 2,4-D - OPC11-1600; KP (S8) 3.0 μM 2,4-D - OPC02-200; PC (S6-S8) 10 μM 2,4-D - OPC07-225; and PC (S5-S6) 0.3 μM 2,4-D - OPC14 - 1050 variation was noted as an absence of a band (Table 5.8). Some variation in banding patterns was observed for one subculture and was not detected again in subsequent subcultures. Other changes in genomic DNA profile were noted for two or more successive subcultures (grouped together in Table 5.7). Examples of variant DNA profiles generated for cultivar PC by different primers are shown in Plate 5.5.

RAPD analysis of embryogenic suspensions: Cultivar-specific polymorphic markers useful in identifying genetic variation occurring in embryogenic suspension cells are listed in Table 5.6. Polymorphic bands generated by each primer were generally noted as the addition of strong or intense bands.

Table 5.3 List of primers used, the number of DNA bands amplified for asparagus(*Asparagus* officinalis L.) cultivars Aspiring, Karapiro and Pacifica, their molecular size range and the number of intense and strong bands generated. '*' in table indicates no detectable amplified products.

Primers	Cultivars								
	Aspiring Amplified products			Karapiro Amplified products			Pacifica Amplified products		
	Total no. of amplified bands	Size (bp)	No. of intense and strong bands	Total no. of amplified bands	Size (bp)	No. of intense and strong bands	Total no. of amplified bands	Size (bp)	No. of intense and strong bands
OPC-01	12	100-1900	10	7	200-1900	5	11	150-2000	4
OPC-02	12	100-2000	8	9	150-1900	4	13	100-1800	7
OPC-07	10	100-1500	6	11	200-200	5	7	150-1400	5
OPC-08	9	400-900	6	5	100-1600	5	7	550-2000	5
OPC-09	6	100-1200	4	7	150-1200	3	7	300-1600	6
OPC-10	7	200-1700	3	6	200-950	2	7	150-1600	5
OPC-11	2	700-1600	2	4	600-1600	2	7	600-2000	7
OPC-12	15	400-1500	8	14	400-1600	12	8	400-1400	12
OPC-13	6	700-1500	4	*	*	*	12	100-2000	8
OPC-14	7	300-1600	5	8	550- 1600	5	12	450-1800	7

Table 5.4 Cultivar specific polymorphic markers useful for identifying 'Aspiring', 'Karapiro', and 'Pacifica'. Key to table symbols: 'v' = faint bands detected for some PCR recations and not others, '+' = intense bands presents for all PCR reactions but which vary in intensity, '++' = strong bands present for all reactions with the same intensity and '-' = absence of a band.

Markers	Cultivars		
	Aspiring	Karapiro	Pacifica
OPC01-2000	-	-	+
-1800	-	-	++
-1700	-	-	++
-1600	++	-	-
-1200	-	-	v
-1000	+	-	-
-850	-	-	v
-700	+	-	-
-650	-	-	++
-550	++	-	-
-500	-	++	-
-400	-	+	-
-300	+	-	-
-250	v	-	-
-225	-	+	-
-200	-	+	-
-150	++	-	-
-100	+	-	-
OPC02 -2000	++	-	-
-1900	-	+	-
-1700	-	+	-
-1500	-	-	v
-1400	v	-	-
-950	-	-	++
-900	-	v	-
-800	++	-	-
-650	-	+	-
-550	++	-	-
-500	-	++	-
-300	-	v	-
-275	+	-	-
-150	-	v	-
OPC07-2000	-	v	-
-1500	v	-	-
-1100	-	v	-
-1000	++	-	-
-950	-	+	-
-800	-	+	-
-500	++	-	-
-400	-	v	-
-375	-	-	+
-325	-	-	+
-300	+	-	-
-250	-	+	-
-200	-	v	-
-100	v	-	-

Table 5.4 (continued) Cultivar specific polymorphic markers useful for identifying 'Aspiring', 'Karapiro', and 'Pacifica'. Key to table symbols: 'v' = faint bands detected for some PCR recations and not others, '+' = intense bands presents for all PCR reactions but which vary in intensity, '++' = strong bands present for all reactions with the same intensity and '-' = absence of a band.

	Cultivars		
Markers	Aspiring	Karapiro	Pacifica
OPC08-2000	-	-	++
-1800	v	-	-
-1600	-	+	-
-1400	+	-	-
-1300	-	-	v
-950	+	-	-
-850	-	-	v
-750	-	-	++
-650	++	-	-
-550	v	-	-
-500	-	-	+
-450	v	-	-
-400	-	++	-
-300	-	++	-
-100	-	+	-
-1600	-	-	+
-1500	-	-	++
-1000	-	v	-
-550	-	-	+
-400	-	++	-
-150	-	++	-
-100	++	-	-
OPC10-1800	v	-	-
-1600	-	-	v
-1500	v	-	-
-1400	v	-	-
-1200	-	-	+
-950	-	+	-
-600	-	v	-
-450	-	-	v
-225	-	++	-
-200	-	++	-
-150	-	-	++
OPC11-2000	-	-	+
-1900	-	-	+
-1200	-	-	+
-100	-	-	v

Table 5.4 (continued) Cultivar specific polymorphic markers useful for identifying 'Aspiring', 'Karapiro', and 'Pacifica'. 'v' = faint bands detected for some PCR recations and not others, '+' = intense bands presents for all PCR reactions but which vary in intensity, '++' = strong bands present for all reactions with the same intensity and '-' = absence of a band.

	Cultivars		
Markers	ASP	KP	PC
OPC12-1600	-	v	-
-950	-	-	+
-425	-	-	v
OPC13-2000	-	*	v
-1800	-	*	v
-1600	-	*	+
-1500	+	*	-
-1400	+	*	-
-1200	-	*	++
-800	v	*	-
-700	+	*	-
-600	-	*	++
-550	-	*	+
-500	-	*	+
-400	-	*	+
-200	-	*	v
-100	-	*	v
OPC14-1800	-	-	v
-1100	-	-	v
-850	-	v	
-800	+	-	-
-700	v	-	-
-500	-	-	+
-350	+	-	-

Table 5.5 Total number of variant DNA profiles revealed by all primers for *Asparagus officinalis* L. cultivar Aspiring, Karapiro, and Pacifica grown on both PGR and PGR-free medium. Treatments: 1-NAA/kinetin, 2- 0.3 μM 2,4-D, 3- 1.0 μM 2,4-D, 4- 3.0 μM 2,4-D, 5- 10 μM 2,4-D.

Cultivar	PGR-medium						PGR-free medium					
	Treatment						Initial PGR treatment					
	1	2	3	4	5	Total	1	2	3	4	5	Total
Aspiring	0	2	0	8	10	20	1	1	6	4	1	13
Karapiro	0	0	0	2	1	3	2	1	0	0	0	3
Pacifica	9	4	9	12	13	47	0	0	0	1	0	1
Total	9	6	9	22	24	70	3	2	6	5	1	17

Table 5.6 Variant DNA bands observed for asparagus (*Asparagus officinalis* L.) embryogenic cells grown in PGR-free liquid medium for one year. Key to table symbols: * = bands present in the original explant but which are absent in samples. '+' = presence of an intense band, '++' = presence of strong band, 'v'= variable bands observed for some Polymerase chain reactions and '-' = absence of a band. ' 'o' = no samples with variant DNA profiles.

Markers (bp)	Initial plant growth regulator treatment				
	NAA/kinetin Subculture no.	0.3 µM 2,4-D Subculture no.	1.0 µM 2,4-D Subculture no.	3.0 µM 2,4-D Subculture no.	10 µM 2,4-D Subculture no.
Aspiring					
OPC02	o	o	o	o	8
-800					+
-750					+
-725					+
-700					+
-650					+
-625					+
-600					+
-550					+
-525					+
-500					+
OPC-07	o	o	7	8	o
-250			+	+	
-200			-	-	
OPC-08	11	11	6-10	8-9	o
-650	-	++	-	-	
-600	-	++	-	-	
-500	-	-	-	+	
-450	-	++	-	+	
-425	-	-	-	+	
-400	-	-	-	+	
-350	+	-	+	+	
-300	+	v	+	+	
-250	-	-	-	+	
-200	-	-	+	-	
-175	-	-	+	-	
-150	-	-	+	-	
-100	-	++	+	-	
-50	-	+	-	-	
OPC-10	o	o	o	8	o
-150				+	
-100				v	

Table 5.6 (continued) Variant DNA bands observed for asparagus (*Asparagus officinalis* L.) embryogenic cells grown in PGR-free liquid medium for one year. Key to table symbols: * = bands present in the original explant but which are absent in samples. '+' = presence of an intense band, '++' = presence of strong band, 'v'= variable bands observed for some Polymerase chain reactions and '-' = absence of a band. ' 'o' = no samples with variant DNA profiles.

	Initial plant growth regulator treatment				
Markers (bp)	NAA/kinetin Subculture no.	0.3 μM 2,4-D Subculture no.	1.0 μM 2,4-D Subculture no.	3.0 μM 2,4-D Subculture no.	10 μM 2,4-D Subculture no.
Karapiro					
OPC-01	7	o	o	o	o
-500	*				
-50	+				
OPC-02	7	o	o	o	o
-500	*				
OPC-11	o	9	o	o	o
-700		++			
-575		++			
-175		++			
Pacifica					
OPC-08	o	o	o	6	o
-400				+	
-200				+	
-100				+	

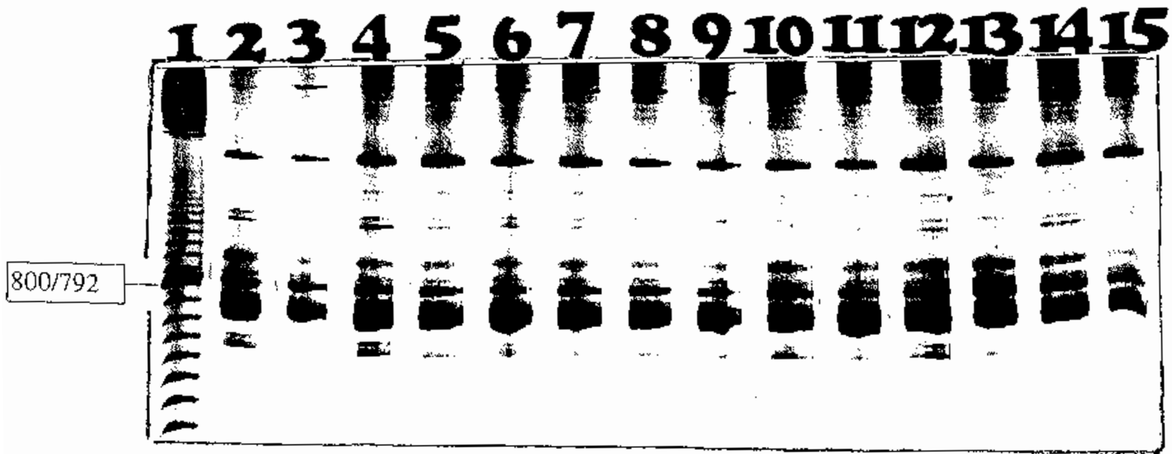

Plate 5.4 DNA profiles of 'Pacifica' plantlets regenerated from somatic embryos derived from calli initiated on medium containing 10µM 2,4-D. Plant DNA was amplified using primer OPC-08 and amplified products were separated on an 8% polyacrylamide gel and silver stained. Lane 1 (100 bp marker); Lane 2 ('Pacifica' explant DNA profile), and Lanes 3-15 (DNA profiles of individual 'Pacifica' plantlets).

5.2.3.5 Effect of PGR treatment and length of time in culture on genetic stability of cultivars:

RAPD analysis of embryogenic calli: Plant growth regulator concentration influenced the number of variant DNA profiles revealed by each primer (Table 5.5). For embryogenic calli maintained on medium containing 2,4-D a total of 24, 22, 9 and 6 variant DNA profiles were generated by all primers for 10, 3.0, 1.0 and 0.3 μM 2,4-D, respectively. Results for calli maintained on medium containing 1.0 μM NAA/ 0.1 μM Kinetin were similar to those obtained for 1.0 μM 2,4-D. For each plant growth regulator treatment the greatest number of variant DNA profiles were generated by cultivar PC (Table 5.5).

The length of time embryogenic calli of each cultivar was maintained on medium containing PGRs did not appear to influence the number of variant DNA profiles revealed by primers (Table 5.8). A cultivar-specific response was noted with the most variant DNA profiles revealed for cultivar PC. The genome of 'KP' appeared to be the most stable, with only 3 variant profiles generated. Most variant DNA profiles were noted for 'PC' samples collected between subculture 4 and 9, those subcultures reflected growth on PGR medium for 4 and 9 months, respectively.

RAPD analysis of embryogenic suspensions: A long-term 'carryover' effect of PGR treatment was not noted for embryogenic cell suspensions transferred from medium containing PGRs and maintained on PGR-free medium. For samples previously maintained on medium containing 2,4-D, the greatest number of variant DNA profiles, generated by all primers, were revealed for cultivar ASP maintained on 1.0 μM 2,4-D, while only one variant DNA profile was noted for 'ASP' maintained on 10 μM 2,4-D (Table 5.5). Similar to embryogenic calli, the length of time embryogenic suspension cells were maintained on PGR-free medium did not influence their genetic stability. More variant profiles were generated for cultivar ASP with the most somatic variants being observed for subculture 8 (Table 5.8).

Table 5.7 Variant polymorphic bands observed in samples of asparagus (*Asparagus officinalis* L.) embryogenic calli maintained on PGR medium for one year. Key to table symbols: '*' = bands present in the original explant but which are absent in samples. '+' = presence of an intense band, '++' = presence of strong band, 'v' = variable bands observed for some Polymerase chain reactions and '-' = absence of a band. 'o' = no samples with variant DNA profiles.

'Pacifica'	Plant growth regulator treatment								
Markers (bp)	NAA/kinetin Subculture no.			0.3 µM 2,4-D Subculture no.	1.0 µM 2,4-D Subculture no.		3.0 µM 2,4-D Subculture no.	10 µM 2,4-D Subculture no.	
OPC01		6	9-12	11	6-9,11	12	8		4
-900		-	-	-	++		-		-
-750		-	-	-	-		-		-
-700		+	-	-	-		-		-
-350		-	-	-	-	*	+		++
-300		++	-	++	-		-		++
-250		-	-	-	v		-		-
-225		-	-	-	-		-		-
-125		-	-	-	++		-		-
-100		++	+	++	-		-		+
OPC-02		8		9	13		o		4
-475		-		-	-				+
-450		-		-					+
-300		-		-	+				++
-250		-		-	+				+
-50		++		+	v				-
OPC07		o		o	13	14	5	6-8	9-10
-900					++	++	-		-
-750					-	-	+		-
-300					+	+	-		-
-275					-	-	-		+
-250					-	-	-		+
-225					-	-	++	*	-
-125					-	-	-		-
-100					-	-	-		+
-50					++	++	-		++
OPC08	4	5	6	o		o	o		o
-1100	-	*	-						
-300	-	++	++						
-250	-	+	-						
-200	+	-	-						
-150	-	++	+						
OPC10		o		o		o	6-15		o
-500							+		
-375							++		
-200							v		
OPC11		o		o		o	8		o
-250							+		
-150							++		
-125							++		
OPC12		o		o		o	o		4-9
-750									++
-700									++
OPC14		o		5-6		o	o		o
-1050				*					

Table 5.7 (continued) Variant polymorphic bands observed in samples of asparagus (*Asparagus officinalis* L.) embryogenic calli maintained on PGR medium for one year. Key to table symbols: * = bands present in the original explant but which are absent in samples. '+' = presence of an intense band, '++' = presence of strong band, 'v'= variable bands observed for some Polymerase chain reactions and '-' = absence of a band. 'o' = no samples with variant DNA profiles.

'Aspiring'	Plant growth regulator treatment							
	NAA/kinetin	0.3 μM 2,4-D	1.0 μM 2,4-D	3.0 μM 2,4-D				10 μM 2,4-D
Markers (bp)	Subculture no.	Subculture no.	Subculture no.	Subculture no.				Subculture no.
OPC01	o	o	o	4	6	8	12	4-12
-1200				-	-	-	-	++
-625				+	-	-	-	-
-550				-	-	-	*	-
-450				-	-	+	-	-
-200				++	-	++	-	-
-75				-	-	+	-	-
-50				-	+	+	-	-
OPC08	o	o	o	8	10	12		o
-150				++	++	++		
-100				++	++	++		
-50				++	++	++		
OPC11	o	12	o		12			12
-1600		*			-			-
-1300					v			-
-1200					v			-
-1100					v			-
-1000					v			+
-900					-			+
-550					+			-
-425					-			+
-400					v			v
-350					v			-
-300					-			++
-200					+			-
-175					+			-
-150					+			-
-125					+			-
-100					+			-
-75					+			-
-50					+			-
OPC13	o	8	o		o			o
-1700		+						

Table 5.7 (continued) Variant polymorphic bands observed in samples of asparagus (*Asparagus officinalis* L.) embryogenic calli maintained on PGR medium for one year. Key to table symbols: * = bands present in the original explant but which are absent in samples. '+' = presence of an intense band, '++' = presence of strong band, 'v'= variable bands observed for some Polymerase chain reactions and '-' = absence of a band. 'o' = no samples with variant DNA profiles.

'Karapiro'	Plant growth regulator treatment				
Markers (bp)	NAA/kinetin Subculture no.	0.3 µM 2,4-D Subculture no.	1.0 µM 2,4-D Subculture no.	3.0 µM 2,4-D Subculture no.	10 µM 2,4-D Subculture no.
OPC02	o	o	o	8	8
-375				-	+
-350				-	+
-325				-	+
-275				-	+
-250				-	+
-225				-	+
-200				*	+
OPC08	o	o	o	6	o
-1300				+	
-700				++	
-500				++	
-175				+	
-150				+	
-75				+	
-50				+	

Table 5.8 Total number of variant DNA profiles revealed for *Asparagus officinalis* L. cultivar Aspiring, Karapiro, and Pacifica by all primers for embryogenic calli and embryogenic cell suspensions maintained on PGR and PGR-free medium respectively.

	Subculture no.											
Cultivar	4	5	6	7	8	9	10	11	12	13	14	15
Calli												
Aspiring	2	0	2	0	4	0	2	0	5	0	0	0
Karapiro	0	0	1	0	2	0	0	0	0	0	0	0
Pacifica	5	5	8	5	8	7	3	3	2	3	2	1
Suspension												
Aspiring	1	0	1	2	5	2	2	0	2	0	0	0
Karapiro	0	0	0	2	0	1	0	0	0	0	0	0
Pacifica	0	0	1	0	0	0	0	0	0	0	0	0

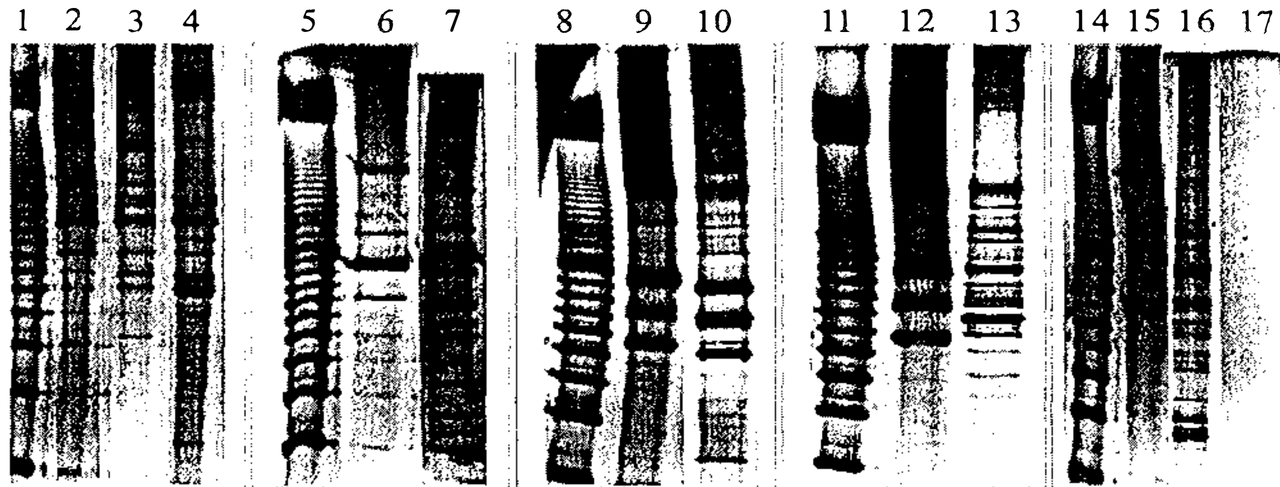

Plate 5.5 Variation in DNA profiles of 'Pacifica' revealed by different primers. Amplified products were separated on an 8% polyacrylamide gel and silver stained. **OPC-01 amplification**: Lane 1(100 base pair marker); Lane 2 ('Pacifica' explant); PGR treatments: NAA/Kinetin Lane 3 (S6); and Lane 4 (S10). Lane 5 (marker); Lane 6 ('Pacifica' explant); PGR treatment: Lane 7 (10 μM 2,4-D, S10). **OPC-02 amplification:** Lane 8 (marker); Lane 9 ('Pacifica' explant); PGR treatment: Lane 10 (NAA/Kinetin, S8); Lane 11 (marker); Lane 12 ('Pacifica' explant); PGR treatment: Lane 13 (10 μM 2,4-D, S10). **OPC-07 amplification:** Lane 14 (marker); Lane 15 ('Pacifica' explant); PGR treatment: Lane 16 (10 μM 2,4-D, S7). Lane 17 (control).

5.2.4 DISCUSSION

RAPD analysis was used to detect somaclonal variation occurring in asparagus cells maintained on both PGR and PGR-free media for 12 months. The results here demonstrate that most primers used generated unique banding profiles for each cultivar and, therefore, could be used to distinguish among them. These results are consistent with an earlier study conducted by the author, where the genome of six asparagus cultivars were screened with 20 primers. Distinct inter-cultivar differences in DNA banding profiles generated by each primer were noted. The sensitivity of RAPD techniques to reveal inter- and intra-cultivar differences have been exploited for the development of markers for genotype identification and to establish linkage to important agronomic characters in asparagus (Khandka *et al.* 1996; Roose & Stone 1996).

In addition to distinguishing between cultivars, this experiment shows that RAPD techniques are sufficiently sensitive to the detection of somatic variation occurring during culture of asparagus embryogenic calli maintained on PGR media and embryogenic suspension cells maintained on PGR-free medium. No changes in the DNA profile of plantlets regenerated from 'Pacifica' somatic embryos were revealed by the primers used. The use of RAPD techniques to reveal changes to genomic DNA structure induced by culture conditions has been reported for a limited number of plant species (Harada *et al.* 1993; Brown *et al.* 1993; Isabel *et al.* 1995; Dan & Stephens 1997). These reports suggest that, while RAPD techniques are useful in revealing gross genetic changes in genomic DNA structure, they may lack the sensitivity to detect smaller changes (Taylor *et al* 1995). Xiao & Christie (1997) report the successful use of RAPDs to characterise camellia cultivars in two sport families. However, Nybom (1994) reports difficulties detecting and characterising natural and induced somatic variants using RAPDs. This difficulty may be due to somatic mutations which arise from single base changes in genomic structure but which do not affect the overall DNA profile and therefore go undetected (Hoepfner *et al.* 1993; Nybom 1994). This is of particular concern for high molecular weight markers where a single base pair (bp) change may not significantly affect the size of the amplified band or its mobility on a gel. It was observed that most of the variant DNA bands detected in this present study

were in the lower molecular weight range (50 and 800 bp). These results give some merit to the assumption that changes in lower molecular weight bands would be more easily detected.

The present study indicates that the ability of RAPD techniques to reveal variation in genomic structure is strongly influenced by the region amplified by specific primers used. This is reflected in the different types, and frequency, of variant DNA profiles observed for similar samples amplified by different primers. Primers OPC-01, -02, -07 and -08 for example, revealed more genomic variation than the other primers. One explanation is that these primers could amplify hypervariable regions (hot-spots for mutations) within the genome. Such regions are known to be genetically unstable and to have higher rates of mutation events than other regions within the genome (McClintock 1984; Flavell 1985; Clegg 1990, Brady *et al.* 1996). As a consequence, any primer that amplifies genomic DNA within these regions should detect changes in DNA structure, which when analysed, should appear as altered DNA profiles. In support of this theory, Natali *et al.* (1995) noted that differential replication of unique repeat sequences in the genome of *Helianthus annuus* L. can influence genome size and contribute to regeneration of genetically altered phenotypes. Likewise, changes in DNA content in regenerated *Pisum sativum* L. plants were associated with changes in unique sequences in the genome (Cecchini *et al.* 1992). Chowdhury & Vasil (1993) also showed that tissue culture conditions produced genetic stress that can result in specific regions of the genome becoming unstable and expressed as a hypervariable segment in both calli and regenerants. These mutational events can be detected by primers that amplify DNA segments within these regions. Collectively these reports may explain the results observed in the present experiments.

More genetically variant DNA profiles were observed for cultivar PC embryogenic calli maintained on PGR medium than for any other cultivar. These results suggests that the genome of cultivar PC may contain hypervariable repeat sequences, such as transposable elements, more susceptible to inter-cellular stress factors, associated with changes in DNA structure, than the genomes of either 'ASP' or 'KP'. Detailed nucleic acid sequencing of each genome, although difficult, would allow direct comparison of

original explants and variant samples to determine which regions of the genome are unstable.

The type and concentration of plant growth regulator used in the culture media and the age of calli are known to influence the frequency of genetically variant plants recovered during regeneration (Kar & Sen 1985; Ghosh & Sen 1992). Generally, rates of somaclonal variation are reported to increase with both increased PGR concentration and length of time in culture (Armstrong & Phillips 1988; Ogura 1990). In a previous study (data not shown) any consistent pattern in changes to DNA profile with either PGR concentration or length of time samples were maintained on PGR medium were not observed. As a result, the current study examined more cultivars and analysis was performed with more primers, enabling the detection of more variation at higher 2,4-D concentrations than in the previous study. Still no pattern related to the length of time in culture was noted. Inconsistencies in the response of embryogenic cells to varying PGR concentrations may be due to several reasons. One is that the majority of reports on somaclonal variation have been based on cytological analysis of individual regenerated plants (Rhodes *et al.* 1986; Armstrong & Phillips 1988), but the present results are based on RAPD analysis of calli. Therefore, there is no way to determine exactly the percent of cells contributing to variation. Some changes in DNA structure may also be lethal and result in cell death. Moreover, genetic changes are small and RAPD techniques or primers used may not have been sensitive enough to detect these changes.

Further studies using more sensitive analytical techniques such as amplified fragment length polymorphisms (AFLPs) (Zabeau & Vos 1992; Vos *et al.* 1995), microsatellite analysis (Rafalski & Tingey 1993; Rongwen *et al.* 1995), cleavase fragment length polymorphism (CFLP) (Boehringer Mannheim Website 1997; CFLP Scan™ Kit Website 1997) or single stranded conformation polymorphism (SSCP) (Bioproducts Website 1997; Mutant Detection Website 1997) are required to detect small changes to genome structure that may be undetected by RAPD analysis. These techniques can also be used to determine if the observed changes are a true reflection of DNA sequence alterations caused by culture conditions, changes in DNA methylation patterns to silence potentially lethal genes (LoSchiavo *et al.* 1989; Müller *et al.* 1990), changes occurring in non-coding sequences, or are artefacts of the detection technique.

potentially lethal genes (LoSchiavo *et al.* 1989; Müller *et al.* 1990), changes occurring in non-coding sequences, or are artefacts of the detection technique.

5.2.5 SUMMARY

RAPD technology is a useful tool to reveal inter-cultivar differences in genomic DNA structure in the asparagus cultivars investigated. In addition, the technique is sensitive enough to detect changes to DNA structure induced during embryogenic calli culture on media supplemented with varying PGR concentrations. This study shows that somaclonal variation detected *in vitro* is not reflected in regenerated plants, as no changes in their genomic structure was detected. These results suggest that variant cells produced during somatic embryogenesis are selected against in culture and die, or may result in precocious germination.

5.2.6 ACKNOWLEDGMENTS

An appreciation is extended to Mr H Neilson for useful technical comments; the Ecology Department for the use of their DyNA Quant Fluorometer and the Animal Science Department for the loan of their gel electrophoresis unit.

Cytological analysis of asparagus embryogenic calli, suspension cells and regenerated plantlets

Abstract

Cytological analysis, using chromosome counts and DNA content analysis, was used to determine the genetic stability of asparagus (*Asparagus officinalis* L.) embryogenic calli, suspension cells, and plantlets regenerated through somatic embryogenesis. Embryogenic calli from cultivars Aspiring, Karapiro, Pacifica, Syn4, Turoa, and UC157 were maintained on callus induction media containing the following PGRs: 0.3, 1.0, 3.0 and 10 μM 2,4-D and 1.0 μM NAA/0.1 μM Kinetin. Embryogenic suspension cells for cultivars Aspiring, Karapiro and Pacifica were maintained on PGR-free media, while plantlets were regenerated from cultivar Pacifica somatic embryos derived from calli initiated on medium containing 10 μM 2,4-D. The basic chromosome number of 20 (2n = 20) remained unchanged for all samples. The DNA content of explants and plantlets was similar, indicating that plantlets were diploid. The experiment was unable to detect somaclonal variation, revealed by altered ploidy level. At least two metacentric chromosomes had secondary constrictions with satellites. Cytological analysis did not detect somaclonal variation detected using RAPD analysis.

Keywords

Asparagus officinalis, chromosome, somatic embryogenesis, plantlets, calli, somaclonal variation, satellite

5.3 EXPERIMENT 6

5.3.1 INTRODUCTION

Historically, chromosome analytical techniques used for the study of plant genomes focused on non-molecular techniques such as evaluating chromosome numbers per cell (Greilhuber 1984; Stace 1989). Today, chromosome analysis of an organism use a more integrated approach combining newer molecular techniques with more traditional cytological approaches (Sharma & Sharma 1994).

Cytological techniques require microscopic examination of prepared cells to obtain information required for chromosome analysis, including:

- light microscopy;
- phase and interference microscopy which has the advantage of being able to quantify phase changes in the material being observed, measurements of mass per unit of cells and changes in DNA and protein content in the nucleus can be ascertained with precision;
- fluorescent microscopy which can be used to obtain quantitative measurements of cellular contents such as DNA;
- both microspectrophotometry and microspectroflurometry may be used to make quantitative measurements of chromosome constituents in the whole nuclei as well as in intact organelles (Berlyn & Cecich 1976; Lawrence & Possingham 1986; Berlyn *et al.* 1990). The development of immunocytochemistry, biotinylated and fluorescent probes has added to the usefulness of these, and other, optical techniques in plant tissue culture. Cytophotometric analysis was used to evaluate *in vitro*-induced somatic variation for a number of plant species (Rhodes *et al.* 1986; Mo *et al.* 1989; Cecchini *et al.* 1992);
- flow cytometry which is used for sorting and quantifying chromosomal data at the microscopic level;
- chromosome image analysis which involves the microelectronic analysis of chromosomes; and

- confocal microscopy which produces three dimensional images of chromosomes through the combined use of confocal scanning optical microscopy, laser scanning and immunofluorescent staining (Sharma & Sharma 1994).

Common techniques used to analyse the genetic stability of plantlets regenerated through micropropagation involves determining if the chromosome number, or structure, of the donor plant was maintained throughout culture (Parfitt & Arulsekar 1987). Evaluation of regenerated plantlets has revealed that variation in chromosome number and structure, single gene mutations in the nucleus and changes in cytoplasmic DNA (both mitochondrial and chloroplast) occurs in culture (Evans *et al.* 1984). Regenerated plantlets can be classified as diploid, polyploid, aneuploid and chromosome number chimeras (mixoploids), based on the type of chromosomal changes that occur during *in vitro* culture (Ogura 1990; Yibing & Han 1993). Polyploidy is the most frequently detected chromosomal abnormality (Geier 1991).

The nuclear DNA content estimated for several plant species, is constant and characteristic for each species (Swift 1950). DNA amounts are expressed as '*C*'-values, and the *C*-value (1*C*) for any genome is the DNA content of the unreplicated haploid chromosome complement (Bennett & Smith 1976). *C*-values are estimated by using nuclei, of known ploidy, which are not in the *S*-phase of mitotic division. The estimates of DNA amounts are often expressed as a mean content per cell. Therefore, in a meristem containing diploid cells the mean DNA content per cell will be between 2 and 4*C*. Because estimated nuclear DNA amounts are specific for plant species, evaluation of changes in nuclear DNA content of *in vitro*-cultured cells can be used to monitor the occurrence of somaclonal variation during culture.

Cytological analysis revealed ploidy variation in calli and regenerated plantlets of several plant species including, asparagus plantlets regenerated from somatic embryos (May & Sink 1996), Anthurium (Geier 1988) and sugarcane (Heinz & Mee 1971).

In this study, the genetic stability of embryogenic calli maintained on medium containing PGRs for 1½ years, embryogenic cells initiated on medium containing PGRs, but maintained in PGR-free medium for 1 year, and plantlets regenerated from somatic embryos were evaluated by cytological analysis. Basic chromosome counts and cytophotometric measurements of nuclear DNA content were used.

5.3.2 MATERIALS AND METHODS

5.3.2.1 Donor plant material

Three cultivars, Pacifica (PC), UC157 (UC) and Syn4 (SN), used in this investigation were maintained at the greenhouse units at Massey University. Both 'UC' and 'SN' were established from seed while 'PC' was established from clonal material. Cultivars, Aspiring (ASP), Karapiro (KP) and Turoa (TU) were obtained and maintained as tissue-cultured liners from Aspara Pacific, New Zealand. Root tip samples of storage roots from each cultivar were selected for cytological analysis.

5.3.2.2 Calli and embryogenic cells

Calli produced during the callus induction experiment (Chapter 3, experiment 1) and maintained on MS media containing 0.3, 1.0, 3.0, and 10 μM 2,4-D and 1.0 μM NAA/ 0.1 μM Kinetin for 1½ years were used. Calli were subcultured every 3 weeks and samples collected for cytological analysis. Embryogenic cells collected during the somatic embryo induction and development experiment (Chapter 3, experiment 2) were used for cytological analysis. Calli samples were randomly selected from different areas of the callus mass and were collected after each subculture.

5.3.2.3 Regenerated plantlets

Three-month-old acclimatised plantlets regenerated from 'Pacifica' somatic embryos were used. Plantlets were maintained in the greenhouse units at Massey University. Root tip samples of storage roots from 50 'Pacifica' plantlets were selected for cytological analysis. These plantlets were the same used during RAPD analysis (Chapter 5,

experiment 5). All plantlets were derived from calli initiated on 10 μM 2,4-D, 10 plantlets were derived from calli also treated at -15^{0}C.

5.3.2.4 Determination of ploidy level

Plant tissue were pretreated with 0.05% colchicine (Sigma) for 30 min, 1, 1½, 2, 2½, 3, 3½ and 4 hr, fixed in ethanol:glacial acetic acid (3:1) at 4^{0}C overnight and stored in 70% ethanol until required. Sample preparation for chromosome counting was as follows: plant tissue was softened in 1N HCl at 60^{0}C for 10 min in the dark, hydrolysis was terminated by immersing tissue in distilled water for 5 min, tissue were stained in Feulgen (BDH) stain for 2 hr in the dark, squashed in 45% acetic acid and viewed with a light microscope. Two slides were prepared per sample and chromosomes from 5 cells were counted per slide.

5.3.2.5 Determination of nuclear DNA content

Two grams of calli or young actively growing spear tissue from explants and regenerated plantlets were selected and thinly sliced. Prepared plant tissue was incubated overnight at 27^{0}C in 25 ml of enzyme solution (0.6 M D-mannitol pH 5.5, 10 mM $CaCl_2$, 0.2 % w/v pectinase (Sigma) and 0.5% w/v cellulase (Sigma) were filter sterilised through a 0.2 μm filter (Sartorius)). Petri dishes containing samples were kept on a rotary shaker at 30 rpm. Protoplasts were collected by filtering samples through a 32μm steel mesh filter into a conical tube kept on ice, the filtrate was centrifuged at 800 rpm for 5 min at 4^{0}C. The supernatant was removed and the protoplasts suspended in 1 ml Galbraith's buffer (45 mM $MgCl_2$, 20 mM MOPS (BDH), 0.1% triton X-100 and 30 mM sodium citrate adjusted to pH 7). Protoplasts were lysed in this buffer, nuclei were collected by centrifuging at 200 rpm for 3 min at 4^{0}C. The supernatant was removed and the nuclei stored at -20^{0}C in 500 μl glycerol containing 50μg/ml propidium iodide (PI) (Sigma). Samples were shipped (on ice) to HortResearch Cytoflowametric Laboratory, Auckland for analysis of DNA content.

5.3.2.6 Flow cytometry

The following is a brief description of the flow cytometric procedure used (I. O'Brien per. comm. 1997): The EPICS Elite *ESP* flow cytometer (Coulter Electronics, Hialeah, Florida, USA) was used. Linearity of the flow cytometer was ensured by using linearity beads (Coulter Electronics) on a monthly basis, and by using plant standards of known DNA content (*Hordeum vulgare* L. cv. Sultan, 11.12 pg/2*C* nucleus and *Triticum aestivum* L. cv. Chinese Spring, 34.85 pg/2*C* nucleus) prepared in the same way as the unknown samples.

Prior to analysis, a 200 μg/ml PI solution was run through the flow cytometer for 5 min to saturate the 200 μm silicone tubing to prevent loss of the fluorochrome from the nuclei to the tubing during analysis. Room temperature was maintained constant (22 ± 1°C) to minimise laser fluctuations. To monitor any variation in laser intensity, samples were spiked with Immunocheck beads and only variation between fluorescence channels 918 and 923 was permitted. If there was any variation from this then the photomultiplier tube (PMT) voltages were adjusted to return settings to within the predetermined parameters.

For nuclear analysis PI was excited with 15 mW beam of an argon ion laser (488 nm), with the standard filter configuration of the EPICS Elite *ESP* flow cytometer. Cells were collected based on either their log PMT4 versus FS (forward scatter) to eliminate chloroplasts, or on their FS versus SS (side scatter) to identify apoptotic cells. The discriminator was set at 150 of the PMT4 signal to also eliminate chloroplasts from analysis. When possible, at least 10 000 nuclei were analysed with clumped nuclei eliminated by gating on peak vs. integral fluorescence of the PI signal.

The instrument was cleaned with CoulterClenz for 3 min (Coulter Electronics) at the end of analysis, followed by 2% hypochlorite solution for 3 min, then finally flushing and purging with CoulterClenz for 6 min. Isoton II (Coulter Electronics) was used as the sheath fluid.

5.3.3 RESULTS

5.3.3.1 Chromosome counts

All samples analysed contained the normal chromosome number for asparagus (2n = 20) (Plate 5.6). Treatment of samples for 3 to 3½ hr in colchicine resulted in approximately 70% of chromosomes at metaphase (Plate 5.6a). Incubation of tissue in colchicine for less than 3 hrs resulted in approximately 80 to 90% of chromosomes at prophase and late prophase (Plate 5.6a and b). Two metacentric chromosomes with secondary constrictions and satellites were also observed (Plate 5.6a). In Plate 5.6b, these two satellites can be seen separated from their chromosomes and could mistakenly be counted as two extra chromosome (2n = 22).

5.3.3.2 DNA content

All explants and plantlets regenerated from somatic embryos were diploid (2-4*C* depending on the phase of mitotic division). Samples prepared from calli and suspension cells did not have enough viable nuclei to be analysed; therefore, no data is available.

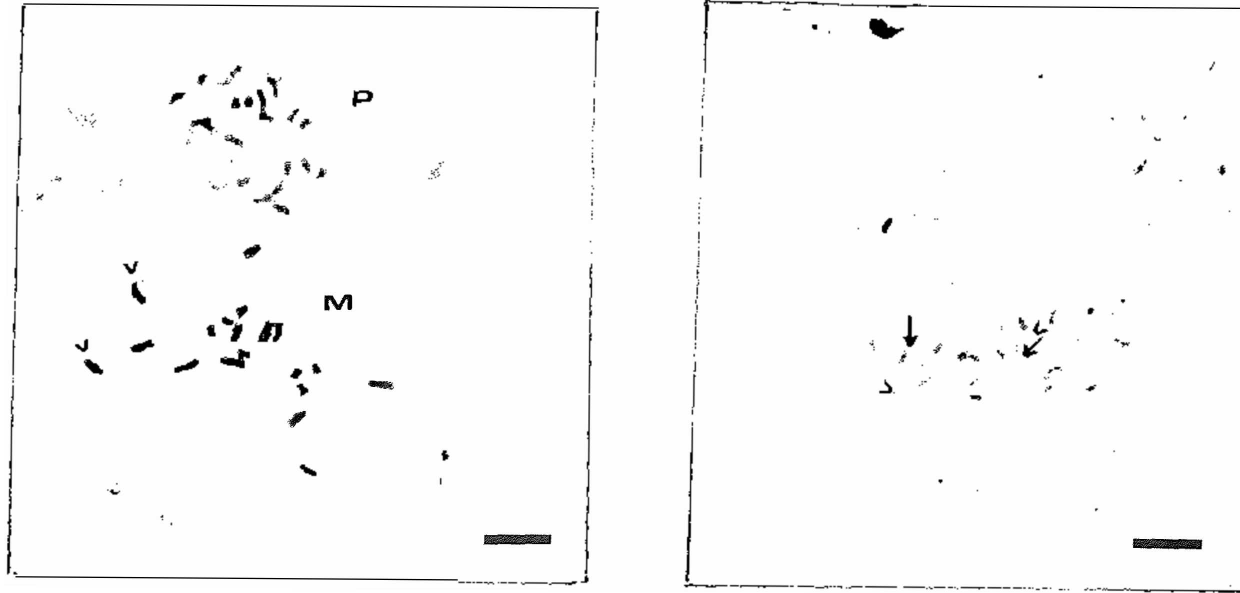

Plate 5.6 *Asparagus officinalis* L. chromosomes (2n = 20).

(a) Chromosomes at metaphase (M). Two metacentric chromosomes with satellites (indicated by arrow head). Late prophase (P) chromosomes. Bar = 33 μm

(b) Late prophase chromosomes, satellites (indicated by 'v') have broken off from the metacentric chromosomes and appear as two extra chromosomes (2n = 22). Metacentric chromosomes from which both satellites originated are indicated by an arrow. Bar = 33μm

5.3.4 DISCUSSION

Chromosome counts and DNA content measurements were used to assess the genetic stability of asparagus (*Asparagus officinalis* L.) embryogenic calli, cells and plantlets regenerated from somatic embryos. Incubation in colchicine for 3¼ hr was ideal for preparation of asparagus root tips and calli for chromosome counting. No variant chromosome numbers were detected for samples analysed. The results for regenerated plantlets confirm the author's earlier study with RAPDs which also indicated that regenerated plantlets were genetically stable. DNA content measurements also confirmed that acclimatised 'Pacifica' plantlets were diploid. The poor results from calli and suspension samples indicate that the protoplast isolation and lysis procedure needs modification to enhance nuclei recovery from these tissues.

Cytological analysis has practical worth for analysing individual plantlets; however, it may be limited for analysis of calli which can comprise several cell types (see later section on callus characterisation). Results of cytological analysis, and RAPD analysis, of calli and suspension cells, are based on those cells selected to be analysed and may not be representative of the total population of cells. This problem was avoided by randomly selecting samples from different areas of calli. Although unable to detect changes in ploidy level, cytological analysis has been used to detect somaclonal variation in the asparagus genus. For example, *A. racemosus* (Kar & Sen 1985); *A. cooperi* (Ghosh & Sen 1992); and *A. officinalis* (Reuther 1990; Guangyu 1996). Geier *et al.* (1992) successfully used cytomorphometric analysis to examine quantitative variation in nuclear DNA content of explant, calli, and cell suspensions of Poinsettia. Similar to the results of this study, Geier found plantlets regenerated from embryogenic cultures to be genetically uniform and similar to the explant. Additionally, although a high degree of quantitative variation in nuclear DNA content (ranging from 2*C* to 16*C*) of calli and cell suspensions were detected, DNA content of embryogenic cell masses were 2*C* (similar to the explant).

Earlier an allusion was made to the fact that cytological techniques fail to detect subtle changes to genome structure. This was confirmed by the present study, since genetic

variation revealed by RAPD analysis was not detected by chromosome analysis of samples collected at the same time.

5.3.5 SUMMARY

Chromosome counts and DNA content analysis did not reveal variation in ploidy level of samples of asparagus embryogenic calli, suspension cells and regenerated plantlets investigated. Cytological analysis of regenerated plantlets confirmed results from the earlier study using RAPD that plantlets regenerated from 'Pacifica' somatic embryos are genetically stable. Cytological analysis was less sensitive than RAPD analysis at detecting changes to asparagus genomic structure.

5.6.6 ACKNOWLEDGEMENTS

An appreciation is extended to Dr R Rowland and E Nickless for their assistance and technical advice during this experiment, and I O'Brien from HortResearch Cytoflowametric Laboratory, Auckland for DNA content analysis.

Detection of extracellular proteins secreted by asparagus embryogenic cells in suspension cultures

Abstract

The effect of plant growth regulator (PGR) concentration and length of time in culture, on the expression of extracellular proteins secreted into the medium of asparagus (*Asparagus officinalis* L.) embryogenic cell cultures was investigated using cultivars Aspiring, Karapiro, Pacifica, and Turoa. Embryogenic calli were initiated on Murashige and Skoog (MS) medium containing 0.3, 1.0, 3.0 and 10 μM 2,4-D plus 1.0 μM NAA/0.1 μM Kinetin. Embryogenic cell suspensions were then obtained by transferring embryogenic calli into PGR-free liquid medium. Extracellular proteins were isolated from the media of actively growing embryogenic suspension cultures and analysed by gel electrophoresis. Total extracellular proteins between 14 and 97 kD were detected in the culture medium of cultivars and could be used to distinguish each cultivar. High molecular weight extracellular glycoproteins greater than 97 kD were also detected. In addition, both the concentration of PGR used to initiate embryogenic calli and the length of time cells were maintained on PGR-free medium were shown to influence the expression of extracellular proteins. Extracellular proteins (18, 34, 43, and 66 kD for cultures derived from calli grown on 3.0μM 2,4-D, and 40, 50 and 70 kD for cultures initiated from calli grown on 10 μM 2,4-D) expressed by 'Aspiring' embryogenic cells growing in PGR-free medium were inhibited when the cells were transferred into medium containing PGRs. Further studies are required to determine the effect of these extracellular proteins on somatic embryo development.

Keywords

Asparagus officinalis, micropropagation, somatic embryogenesis, plant growth regulators, glycoproteins

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An abbreviated report on this experiment was presented at the IX International Asparagus Symposium, July 15-17, 1997 and was accepted for publication in the conference proceedings and will appear in Acta Horticulturae.

5.4 EXPERIMENT 7

5.4.1 INTRODUCTION

Suspension cultures of asparagus (*Asparagus officinalis* L.) somatic embryos offer opportunities for large-scale production of select asparagus clones (Reuther 1996) as well as providing a system to investigate biochemical changes that affect somatic embryo development. In the past, changes in morphological and physiological characteristics have been used to investigate somatic embryogenesis at the cellular level. Recent attention has focused on biochemical changes occurring during embryogenesis. Biochemical studies give some insight into regulatory mechanisms involved in the induction of embryogenic cells from competent somatic cells and the maintenance of embryogenic gene expression over prolonged periods. Somatic embryo development can be followed by investigating patterns of accumulation of embryo-specific lipids (Dahmer *et al.* 1991), proteins (Galau *et al.* 1986; Shoemaker *et al.* 1987; Misra *et al.* 1993) and carbohydrates (Lindsey & Topping 1993; Sharma & Thorpe 1995). While research has largely concentrated on determining the intracellular biochemical changes affecting somatic embryogenesis (Akazawa & Hara-Nishimura 1985; David *et al.* 1989), few reports are available on extracellular events affecting this process and, in particular, the effects of extracellular proteins that either promote or inhibit somatic embryogenesis.

Extracellular proteins have been isolated from media containing embryogenic cultures of different plant species including: carrot (LoSchiavo *et al.* 1990), grapevine (Coutos-Thevenot *et al.* 1992), and *Citrus* (Gavish *et al.* 1992). Some extracellular proteins have been shown to be linked with *in vitro* somatic embryo development. Domon *et al.* (1994) demonstrated that two extracellular glycoproteins isolated from Caribbean pine embryogenic cultures were associated with early-stage embryogenesis and were not detected in non-embryogenic cell cultures. The pattern of extracellular protein accumulation is influenced by the presence of PGRs, such as auxins and cytokinins, in the growth medium (Satoh & Fujii 1988; Mo *et al.* 1996). Normally the secretion of some extracellular proteins has coincided with the removal of auxin from the medium. Coutos-

Thevenot *et al.* (1992) recorded the appearance of specific glycoproteins within 5 days of transfer of grapevine embryogenic suspension cells to PGR-free medium.

No reports of extracellular proteins secreted by asparagus somatic embryogenic cell cultures were found to date. This study investigated the occurrence of extracellular proteins secreted during growth of asparagus somatic embryogenic suspension cultures, the influence of plant growth regulators and the length of time of culture on the secretion of extracellular proteins.

5.4.2 MATERIALS AND METHODS

5.4.2.1 Plant material

Three asparagus cultivars Aspiring (ASP), Karapiro (KP) and Turoa (TU) were obtained, and maintained, as tissue-cultured liners from Aspara Pacific Ltd., Christchurch, while the cultivar Pacifica (PC), originally sourced from Circle Pacific, Hawkes Bay, was maintained in a greenhouse at Massey University.

5.4.2.2 Embryogenic cell culture

Protocols and conditions for the production of embryogenic cell cultures were the same as for Chapter 3, experiment 2. Cells were subcultured onto fresh PGR-free embryo induction (EI) medium every 4 weeks; the culture medium was collected at each subculture and used for protein analysis. The extracellular protein profiles of 'Aspiring' embryogenic cells grown on medium containing 3.0 and 10 μM 2,4-D and PGR-free medium was compared. Embryogenic cells grown on EI medium were transferred to medium containing PGRs for 2 weeks and the cell extract evaluation. These cells were then transferred back onto EI medium for 2 weeks and the protein profile of the extracellular filtrate analysed.

5.4.2.3 Extracellular protein extraction

Twenty-five to thirty millilitres of pooled liquid growth medium were separated from a heterogeneous population of embryogenic cells by first filtering through a coarse filter (sterile muslin cloth), followed by centrifugation at 6000 rpm for 15 minutes. The medium was further clarified by filtering through a 0.45μM pore size nitrocellulose filter (Sartorius), freeze dried and resuspended in 1ml of sterile distilled water. Proteins were precipitated overnight by addition of 2.5 volumes of absolute ethanol at 4^0C, and recovered by centrifugation at 10 000 rpm for 5 minutes, the supernatant was removed, proteins dried and stored at -20^0C until required. Proteins were resuspended in 100 μl of 1x phosphate buffer saline (PBS) (Sambrook *et al.* 1989b) and analysed by gel electrophoresis.

5.4.2.4 Protein content determination and gel electrophoresis

Protein content was determined by the method of Bradford (1976), between 3-6 μg of protein was loaded per well. Mini Protean™ II dual slab cells (BioRad) were used for gel electrophoresis. Gels of 12% polyacrylamide were run for 45 minutes at 200 V. To avoid over-heating of the buffer, elctrophoresis was performed at 4°C. BioRad's low range molecular weight standards were used. Gels were analysed by a modified silver staining technique Dunn (1993). Gels were fixed twice for 45 min in a solution containing 40% (v/v) ethanol, and 10% (v/v) acetic acid, rinsed for 10 min in 20% (v/v) ethanol, followed by 10 min in distilled water. Gels were then sensitised for 1 min in a solution containing 0.3 g/l sodium thiosulphate pentahydrate and rinsed for 1 min under a continuous flow of distilled water. Silver staining was performed for 30 min in a freshly prepared solution of 2 g/l silver nitrate and 250 μl/l of 37% (w/v) formaldehyde, gels were rinsed for 10-20 sec in distilled water, developed for 15-30 minutes in a solution containing 30 g/l potassium carbonate, 250 μl/l of 37% formaldehyde and 10 mg/l sodium thiosulphate pentahydrate. Development was terminated by soaking gels in a solution containing 50 g/l Tris, 20 ml/l acetic acid. Gels were photographed immediately after development and stored, at room temperature in ziplock bags containing water.

5.4.2.5 Extracellular glycoprotein detection

A Protean™ II slab cell elctrophoresis system (Biorad) was used. Normally, 100 - 130 μl of protein, containing (10 - 15 μg of protein), was loaded per well. Gels of 12 % polyacrylamide were run for 3-4 hr at 150 - 175 V. After electrophoresis, gels were stained overnight in a solution containing: 0.1% (w/v) coomassie brilliant blue (CBB) dye in 2 % (v/v) phosphoric acid, 10% (w/v) ammonium sulphate, and 20% (v/v) methanol. This CBB staining technique is reported to be highly sensitive, detecting 0.5 - 1.0 ng protein per band (Dunn 1993). Gels were washed 1 min in 25% methanol and photographed. For glycoprotein staining, CBB stained gels were destained overnight in a solution containing: methanol: distilled water: acetic acid (9:9:2 v/v/v). A modified silver staining technique was used (Dunn 1993). Destained gels were washed 5 min in distilled water and incubated 60 min at 4^{0}C in 0.2% (w/v) aqueous periodic acid, rinsed 3 times for 10 min each in 1L distilled water and soaked 3 hr or overnight in 1L distilled water. Silver staining was performed for 15 min in silver diamine solution of 1.4 ml concentrated ammonium hydroxide, 21 ml of 0.36% (w/v) sodium hydroxide, stirred vigourously and then 4 ml of 19.4% (w/v) silver nitrate *slowly* added. When the brown transient precipitate cleared, the solution was made up to 100 ml with distilled water. After silver staining, gels were rinsed for 2 min in distilled water and developed for 5-30 min in a solution of 0.05% (w/v) citric acid and 0.019% (w/v) formaldehyde. Gels were washed 1 min in distilled water and photographed. This silver staining technique for glycoproteins is reported to detect approximately 0.4 ng bound carbohydrate per band (Dunn 1993).

5.4.3 RESULTS

5.4.3.1 Cultivar specific extracellular proteins

Total extracellular proteins between 14 and 97 kD were detected in the culture media of all cultivars (Fig 5.1). Only a few proteins were detected with molecular weights below 14 kD and above 97 kD (data not shown). The type of extracellular protein secreted

varied between cell lines of different cultivars. These inter-cultivar differences were observed for each PGR treatment used in the callus initiation protocol (Fig 5.1). Production of extracellular proteins was variable within some cell culture, this variability was associated with the production of freely suspended cells in the culture medium.

5.4.3.2 Effect of plant growth regulator treatment on extracellular protein profile

Extracellular protein profiles also varied with the type and concentration of PGR used to initiate embryogenic calli (Fig 5.2). Gel photographs are in Appendix Plate 5.7. Some protein bands showed intra-cultivar similarities at all PGR treatments. For example, for cultivar ASP (Fig 5.2a), protein bands of approximately 60 and 87 kD were present in the medium of all cultures regardless of their PGR treatment, whereas cell lines developed from callus maintained on 1.0 μM NAA/ 0.1μM kinetin, 0.3, 1.0 and 10 μM 2,4-D produced proteins of 40 kD. Protein bands of 29 and 23 kD were detected in the media of cells pre-treated with 1.0 and 3.0 μM 2,4-D. All other bands were different for each PGR treatment.

An 80 kD protein was secreted by all 'TU' (Fig 5.2b) cell lines irrespective of PGR treatment. Two proteins of approximately 18 and 60 kD were produced by 'TU' cell lines derived from calli pre-treated with 1.0μM NAA/0.1μM kinetin and 0.3, 1.0 and 3.0 μM 2,4-D. Very little intra-cultivar variation in extracellular protein accumulation was observed for cultivar PC embryogenic suspension cultures. Four bands (50, 35, 29 and 25 kD) were secreted by all cell lines. A notable exception is for cell lines from calli initiated on 10 μM 2,4-D treatment, where bands of 68, 50, 45 and 40 kD were observed. Protein bands of approximately 87 kD were detected in the medium of all 'KP' cell cultures (Fig 5.2c) while 57 and 25 kD proteins were secreted by all 'PC' (Fig 5.2d) cell lines. All other proteins detected for both 'KP' and 'PC' cell cultures were different for each PGR treatment.

Both inter- and intra-cultivar variation in the intensity of protein bands detected were observed for all treatments. While reliable banding patterns were produced in repeated

samples the intensity of the light bands varied with each gel. Production of extracellular proteins was variable within some cell lines.

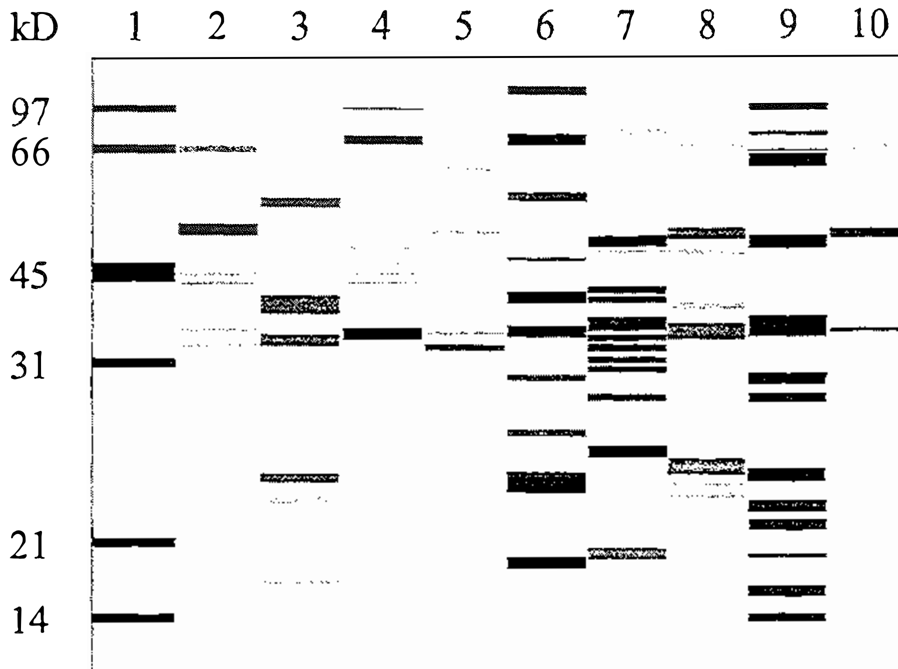

Figure 5.1 Diagrammatic representation of protein profiles drawn from fresh gels showing extracellular protein profiles of asparagus cultivars Aspiring (ASP), Turoa (TU) and Karapiro (KP). Line diagram: Lane 1 SDS-PAGE low range molecular weight marker, lanes 2,3 and 4 cultivars ASP, TU and KP respectively derived from calli treated with NAA/Kinetin; lanes 5, 6 and 7 cultivars ASP, TU and KP derived from calli treated with 0.3 μM 2,4-D and lanes 8, 9 and 10 cultivars ASP, TU and KP derived from calli treated with 10 μM 2,4-D.

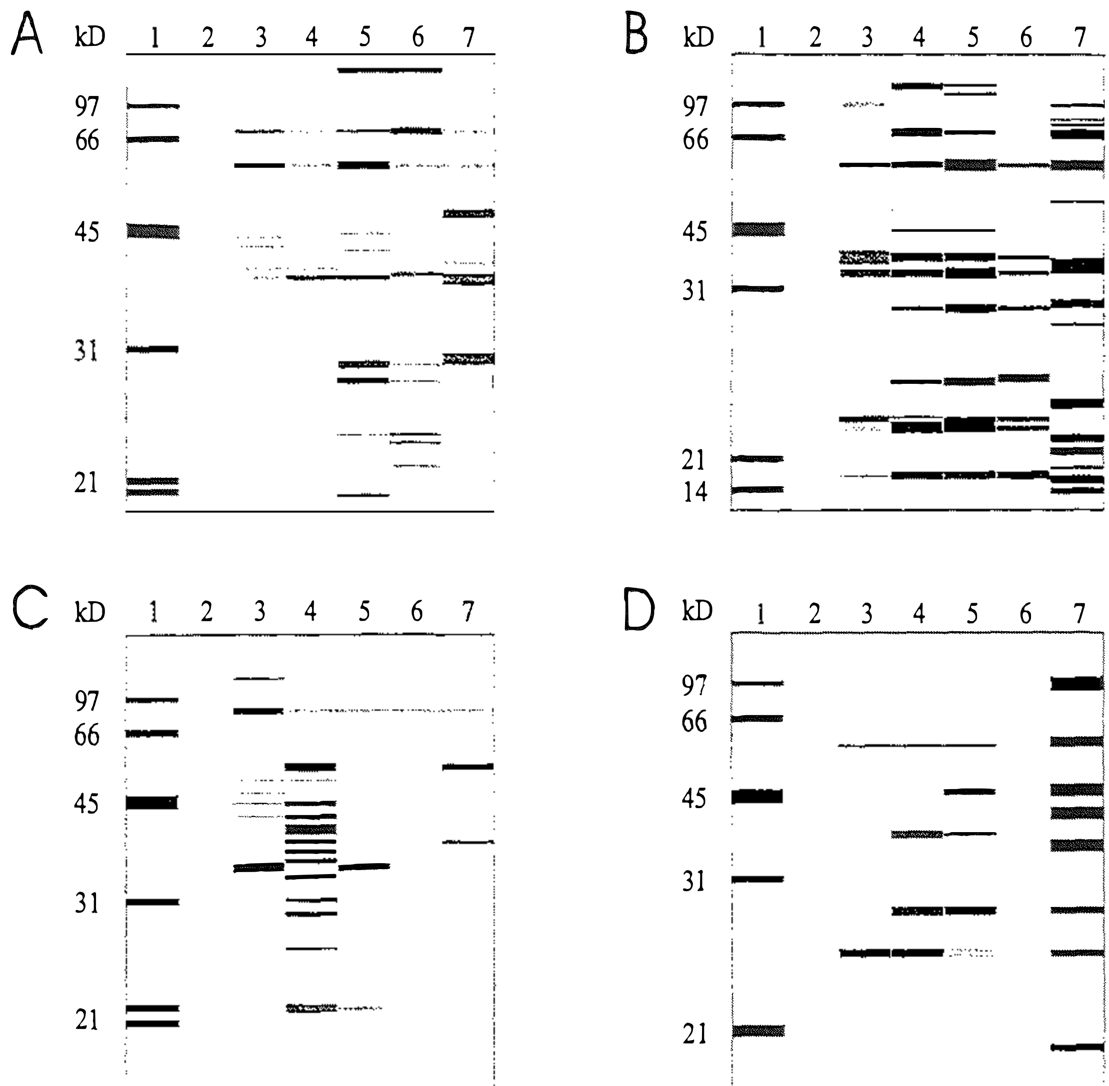

Figure 5.2 Diagrammatic representation of protein profiles drawn from fresh gels showing the effect of type and concentration of PGR on extracellular protein profiles of asparagus embryogenic cultures. Line diagram representation of gel electrophoresis analysis of extracellular proteins isolated from cultivars Aspiring (A), Turoa (B) Karapiro (C) and Pacifica (D). Lane 1 SDS-PAGE low range molecular weight markers; lane 2 control (MS + 6% sucrose); lanes 3-7, proteins extracted from embryogenic cell lines derived from calli treated with NAA/Kinetin, 0.3, 1.0, 3.0 and 10 μM 2,4-D respectively.

5.4.3.3 Stability of extracellular protein profiles over time

The effect of time on expression of extracellular proteins secreted by asparagus embryogenic suspension cultures was investigated by analysing the culture medium after 1 and 3 subcultures. Only cultivar ASP was used in this study. The pattern of extracellular protein expression varied with the length of time embryogenic cells were maintained in culture (Fig 5.3). Normally, more proteins were observed in the culture medium after the first subculture onto PGR-free medium. Different proteins accumulated in the medium of cell cultures pre-treated with 1.0 μM NAA/0.1 μM kinetin and 0.3 μM 2,4-D, after the first and third subculture. Similar protein bands of 40, 37, 35 and 24 kD were detected in the medium of cell lines developed from calli treated with 1.0 μM 2,4-D after each subculture. Likewise, extracellular proteins of 35 kD and 35 and 33 kD were observed for cell cultures derived from 3.0 and 10 μM 2,4-D respectively.

5.4.3.4 Protein profiles of cells grown in the presence of plant growth regulators and PGR-free medium

To determine which extracellular proteins are influenced by the presence of PGRs, cultivar ASP embryogenic cells grown on PGR-free medium were transferred to fresh medium containing PGRs similar to that used to initiate embryogenic calli. More protein bands were observed in suspension cells grown on PGR-free medium than those grown in the presence of PGRs (Fig 5.4). Protein bands of 31 and 39 kD were secreted by the same cells grown in the presence of 3.0 μM 2,4-D and those grown in PGR-free medium, whereas 43 and 37 kD proteins were secreted by cells grown in both 10 μM 2,4-D and PGR-free medium (initiated on 10 μM 2,4-D). Protein bands of 18, 34, 43, and 66 kD were secreted by embryogenic cell lines derived from calli grown on 3.0 μM 2,4-D. When cells were transferred to medium containing 3.0 μM 2,4-D, these proteins disappeared and new proteins of 42 and 50 kD were observed. Embryogenic cells returned onto PGR-free medium secreted extracellular proteins similar to those detected during the first subculture onto PGR-free medium. Similar appearance and

disappearance of putative 'embryo-specific' proteins were observed with embryogenic cells treated with 10 μM 2,4-D.

5.4.3.5 Extracellular glycoproteins

Extracellular glycoproteins were detected only at molecular weights greater than 97 kD (Appendix Plate 5.8). None of the lower molecular weight extracellular proteins were detected as glycoproteins. Similar results were obtained in a preliminary study conducted to determine an appropriate staining technique for extracellular protein detect (data not shown).

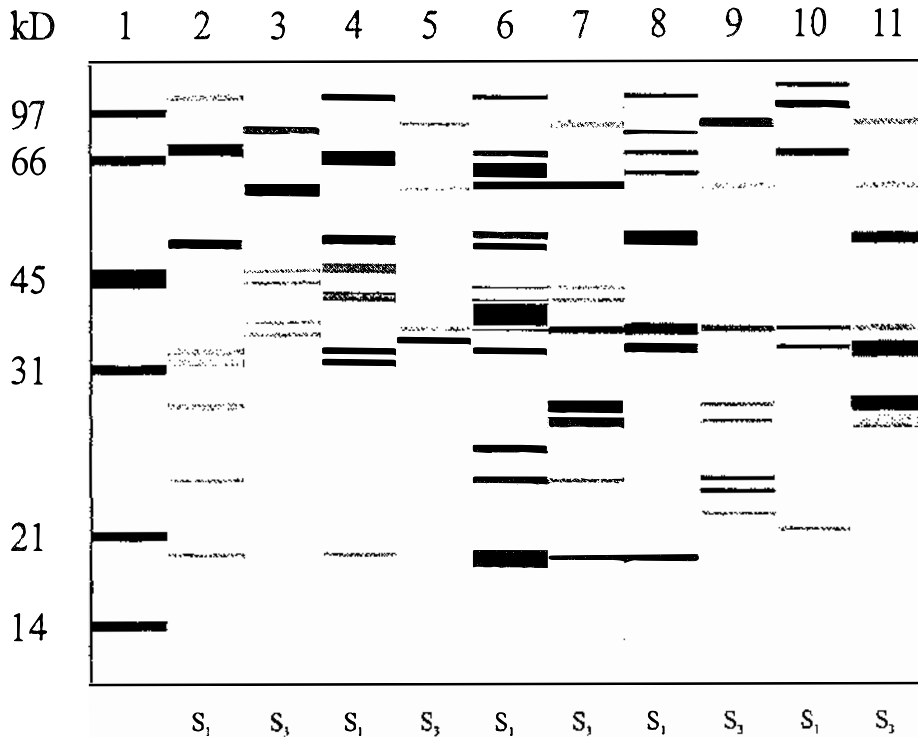

Figure 5.3 Diagrammatic representation of protein profiles drawn from fresh gels showing the effect of time on extracellular protein expression of cultivar Aspiring embryogenic cell cultures. Proteins were analysed from culture media after 1 (S1) and 3 (S3) subcultures on PGR-free medium. Lane 1 SDS-PAGE low range molecular weight marker; lanes 2 (S1) and 3 (S3); lanes 4 (S1) and 5 (S3); lanes 6 (S1) and 7 (S3); lanes 8 (S1) and 9 (S3); lanes 10 (S1) and 11 (S3) represents extracellular profiles observed for cell lines derived from calli initiated on medium containing NAA/Kinetin, 0.3, 1.0, 3.0 and 10 μM 2,4-D respectively.

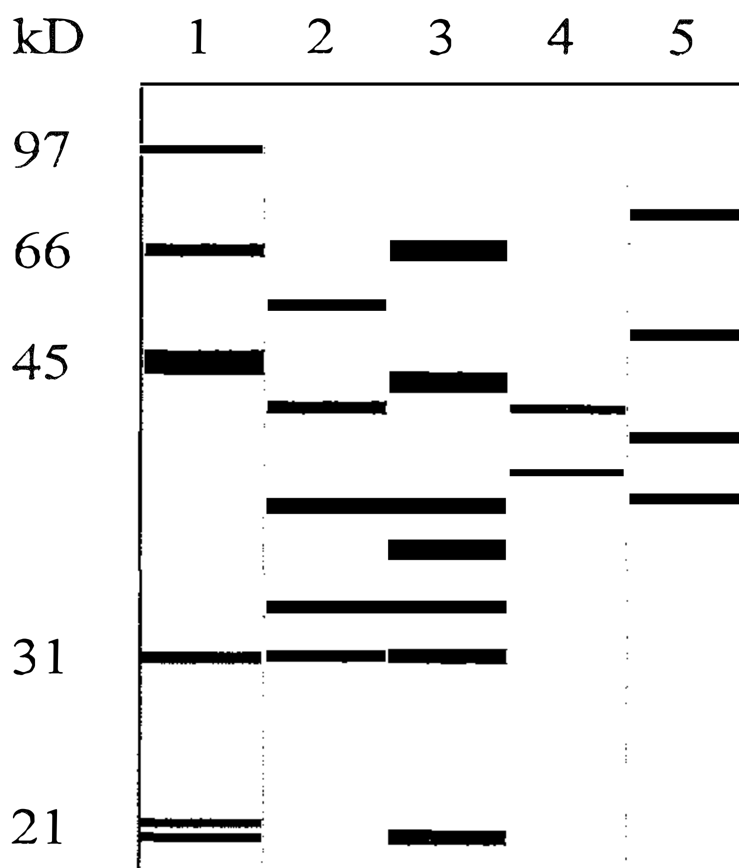

Figure 5.4 Diagrammatic representation of protein profiles drawn from fresh gels showing PGR effects on expression of putative embryogenesis-associated proteins secreted by cultivar Aspiring embryogenic cells. Line diagram showing: Lane 1 SDS-PAGE low range molecular weight markers; extracellular proteins secreted by cell lines derived from calli initiated with 3.0 μM 2,4-D, grown on PGR-free medium and then transferred to medium containing 3.0 μM 2,4-D are represented by lanes 3 lane 2 respectively. Protein profiles of cell lines derived from calli initiated on 10 μM 2,4-D, grown on PGR-free medium and then transferred to medium containing 10 μM 2,4-D are presented in lanes 5 and 4 respectively.

5.4.4 DISCUSSION

Asparagus cultivars could be distinguished by extracellular proteins secreted into the culture medium by actively growing embryogenic cells. Similar results were obtained by Domon *et al.* (1994) who observed similarities in extracellular protein profiles within cell lines of Caribbean pine embryogenic cultures. Specific extracellular glycoproteins in particular were found to be useful in identifying each cell line. Genotypic effects could explain the distinct inter-cultivar differences in protein profile observed. In addition to genotype influences, it was also noted that expression of extracellular proteins was affected by the PGR treatment used to initiate development of embryogenic calli. Of interest is the effect of PGRs on the expression of extracellular proteins associated with somatic embryogenesis. Similar results were reported by Satoh *et al.* (1986), who observed that the appearance or disappearance of two extracellular glycoproteins in the medium of embryogenic carrot cells was regulated by the presence, or absence, of 2,4-D. Plant growth regulator treatments are known to influence both cell line morphology and physiology of embryogenic cells developed during embryogenesis. The long-term effect of PGR treatment was also observed to affect embryogenic cells after several subcultures on PGR-free medium. This effect was a direct result of the type of cell line developed. Mo *et al.* (1996) observed that the morphology of embryogenic cells of *Picea abies* cell lines influenced the expression of extracellular proteins, and that specific proteins could be used to distinguish between cell lines.

Differences in expression of extracellular proteins were observed over time. Variation in protein expression pattern was influenced by several factors including the type of embryogenic cells growing in the medium and developmental regulation of protein expression. More detailed studies are required to further assess the precise influence of culture period and environmental factors on the stability of embryogenesis related extracellular proteins.

Extracellular glycoproteins secreted by embryogenic cells were only detected in the high molecular weight range greater than 97 kD. These data suggest that lower molecular weight extracellular proteins detected did not contain carbohydrate side chains typical of glycoprotein structures (Chambers & Rickwood 1993). This current study was not detailed enough to confidently establish the glycoprotein nature of proteins detected. More studies are required to determine the type of glycosylated side chains attached to core protein structures. This can be achieved by using immuno studies (Satoh & Fujii 1988; Mo *et al.* 1996). In addition, the study should be repeated using larger cell volumes to obtain higher protein concentrations, thereby allowing more analysis to be conducted on the same protein sample. This was not possible in the current study because of shaker space limitations. As it was noted that the type of protein secreted by embryogenic cells is influenced by the length of time cells are cultured, it is, therefore, important to analyse proteins collected from the same sample.

The precise role of extracellular proteins in asparagus somatic embryogenesis has not been elucidated. Some extracellular proteins may play a role in transformation of nucleotides (Ciarrocchi *et al.* 1981), protease activity (Carlberg *et al.* 1984; O'Neill & Scott 1987), cell wall metabolism (de Vries *et al.* 1988), and defence activity (Satoh & Fujii 1988). A 28 kD extracellular polypeptide isolated from *Picea abies* embryogenic suspension cultures by Mo *et al.* (1996) shows a 52-57 % homology with the N-terminal sequence conserved among a protein family known to be involved with plant antifungal mechanisms. In addition, LoSchiavo *et al.* (1990) suggests that some extracellular glycoproteins may be required to initiate the transition from globular to heart stage embryos in carrot cultures. Studies conducted on temperature sensitive carrot cell line variants revealed the function of these extracellular glycoproteins may be dependent on modification of their oligosaccharide side chains.

5.4.5 SUMMARY

Asparagus embryogenic suspension cells secrete extracellular proteins into the culture medium. These proteins are influenced by the type and concentration of PGRs used to initiate development of embryogenic cells. In addition, extracellular profiles examined at any PGR treatment was specific for each cultivar. This suggests strong genotypic

influences on both the type of embryogenic cells developed, and the type of extracellular proteins secreted into the medium. Extracellular proteins unique to embryogenic cells grown on PGR-free medium were also detected. Further studies are required to characterise these extracellular proteins and to elucidate their effect on development of asparagus somatic embryos.

Also investigated was the potential of using extracellular protein profiles to detect somaclonal variation occurring during culture of asparagus somatic embryos. Protein profiles were too variable to be reliably used for this purpose.

Biochemical studies such as those used are essential as they give an indication of some of the factors affecting development of asparagus somatic embryos. Commercial applicability of somatic embryogenesis will be dependent on adapting the system to liquid culture in bioreactors. Therefore, it is important to determine the effect extracellular components, secreted by embryogenic cells, will have on the production of mature somatic embryos capable of producing complete plantlets.

5.4.6 ACKNOWLEDGMENTS

An appreciation is extended to Drs D Harding, K Wurms and D Cook for their useful comments.

SECTION 4

CALLUS CHARACTERISATION

Characterisation of long-term asparagus calli maintained on media containing plant growth regulators

Abstract

Different types of asparagus calli identified after 1½ years growth on callus induction media (MS + 3% sucrose + 1% agar and one of the following plant growth regulators (PGR): 0.3, 1.0, 3.0 and 10 μM 2,4-D and 1.0 μM NAA/0.1 μM Kinetin) were characterised. Six asparagus cultivars, Aspiring, Karapiro, Pacifica, Syn4, Turoa and UC157, were used. Morphological chracterisation of calli was made visually. Anatomical features were characterised with confocal laser scanning microscopy. Embryogenic potential and ability of embryos to convert to plantlets were determined after transfer of calli to liquid embryo induction media (MS + 6% sucrose). The DNA profile of calli and explants were compared using RAPD analysis. Eleven calli types were identified. Overall, individual cultivars produced between 6 to 8 different calli types for all PGR treatments. Plant genotype and PGR treatment influenced the phenotype of calli developed for each cultivar. Different calli types produced ranged from 1 to 4 for any specific PGR treatment. Nodular mucilaginous calli produced more globular embryos than other calli types. Microscopic analysis of globular embryos showed approximately 10% of all embyros simultaneously developed both a root and shoot primordia. For embryos converted to plantlets the root primordia developed about 3 weeks prior to development of shoots. When the root primordia developed, and undifferentiated cell growth occurred at the shoot primordia, no shoot meristem was detected. The low embryo conversion rate could be associated with the lack of shoot meristem development. Cultivar Aspiring and Turoa plantlets were generated from embryos originating from nodular mucilaginous callus. RAPD analysis of calli revealed more variant DNA profiles for calli developed on 3.0 and 10 μM 2,4-D than other PGR concentrations. These variant DNA profiles were associated with the most embryogenic calli types. Further studies are required to determine if this variation is reflected in plantlets regenerated from somatic embryos. The results indicate that, for the six asparagus cultivars investigated in this study, nodular calli or nodular mucilaginous calli have more embryogenic potential than other calli types. These calli

were also noted to produce embryogenic cells in suspension, and could therefore be used to successfully inoculate liquid cultures either for small or large-scale production of asparagus somatic embryos.

Keywords

Asparagus officinalis, somatic embryogenesis, globular embryo, plantlet, maturation, embryogenic suspension, confocal microscopy, RAPD

6.1 CALLUS CHARACTERISATION: A SYNTHESIS OF TECHNIQUES

6.1.1 INTRODUCTION

During *in vitro* culture of callus tissue, the type of calli formed, their organogenic, and embryogenic potential are influenced by plant genotype, explant tissue, media composition, and culture conditions (George & Sherrington 1984). Differences in calli types have been observed in texture, physical properties (Aitchison *et al.* 1977), cellular arrangement, colour, ability to produce suspension cultures, and organogenic and embryogenic potential (George & Sherrington 1984). Some calli types can give rise to other types of callus. For example, Reinert & White (1956) observed that compact calli of *Picea glauca* can give rise to friable calli, the reverse, however, did not occur. The growth and development of various calli have been reviewed by some authors (Street 1974 and 1977; George & Sherrington 1984). These reports are of a general nature and do not focus on calli type specific to embryogenesis, nor do they characterise calli types produced during asparagus somatic embryogenesis.

This section focuses on characterising different types of calli identified during this research. In the callus induction experiment (Chapter 3, experiment 1), it was shown that the phenotype of asparagus calli was influenced by plant genotype as well as the type and concentration of PGR treatment used. It was also noted that the embryogenic potential of each type of callus, developed on callus induction media containing different concentrations of PGRs, varied within and among cultivars.

Although reports are available on the type of embryogenic calli used to produce asparagus somatic embryos, very few of these investigations document the different types of calli that can be produced for one PGR treatment. Levi & Sink (1991a) describe callus developed on induction media (MS + 0.1 mg/l NAA + 0.01 mg/l Kinetin), and transferred to embryo induction media (MS + 1.5 mg/l NAA + 0.01mg/l Kinetin), as 'yellowish and friable'. In another study, Levi & Sink (1991b) characterised callus from asparagus spear sections, developed on callus induction media

(MS + 0.5-2 mg/l 2,4-D + 0-1 mg/l Kinetin) as 'compact, yellowish and mucilaginous'. Callus developed on callus induction media containing MS + 1-3 mg/l NAA + 0-1 mg/l Kinetin was characterised as 'yellowish and friable'. This study demonstrated that compact yellow mucilaginous callus produced more globular embryos than yellow friable callus. Mukhopadhyay & Desjardins (1994a) characterised asparagus embryogenic calli obtained on MS media containing 1 mg/l 2,4-D as 'yellowish, friable and slimy'.

These published reports only briefly describe the type of callus used to produce somatic embryos and do not give an indication of other types of calli produced on asparagus explants when cultured on different callus induction media. In the callus induction experiments (Chapter 3, experiment 1), at least 10 different types of calli were identified. The aim of this investigation was to use techniques from previous experiments to characterise different asparagus calli produced on callus induction media, and to determine their relative embryogenic potential. Characterisation was based on the following criteria:

- phenotype of calli;
- embryogenic potential of calli;
- the type of suspension obtained when calli were grown on embryo induction media;
- type of globular embryos produced;
- maturation and regeneration of globular embryos;
  genetic stability of calli; and
- chromosome number and nuclei DNA content of calli.

Asparagus embryogenic potential is determined by the type of callus produced on explants. The type of callus developed on explants is also genotype dependent. Characterisation of calli produced for each asparagus cultivar would make it relatively easy to identify the type of callus that is more effective at producing somatic embryos and plantlets for each cultivar.

6.1.2 MATERIALS AND METHODS

6.1.2.1 Plant material

Three cultivars, Pacifica (PC), UC157 (UC) and Syn4 (SN), were maintained at the greenhouse units at Massey University. Both 'UC' and 'SN' were established from seed while 'PC' was established from clonal material. Cultivars Aspiring (ASP), Karapiro (KP) and Torua (TU) were obtained, and maintained, as tissue-cultured liners from Aspara Pacific, New Zealand.

6.1.2.2 Callus tissue

Calli produced during the callus induction experiment and maintained on MS media containing 0.3, 1.0, 3.0 and 10 µM 2,4-D and 1.0 µM NAA/ 0.1 µM Kinetin were used. Calli were maintained on callus induction media for 1½ years and subcultured every 3 weeks.

6.1.2.3 Callus phenotype

Callus characterisation was based on visual observation of morphological attributes such as colour and general physical traits. Observations were made using a dissecting microscope.

6.1.2.4 Type of cell suspension obtained on PGR-free media

Calli were transferred to embryo induction media (EI) (MS + 6% sucrose) and observations were made 4 weeks later. Characterisation was based on embryogenic potential, presence of globular embryos, and type of globular embryo. Experimental conditions were similar to those used for experiment 2 (Chapter 3). Anatomical features of embryos were compared using images generated by a confocal laser scanning

microscope (Leica, Heidelberg). Samples were scanned at 512x512 pixels at a depth of 166 μm. Samples for confocal microscopy were either used fresh or stored in 80% ethanol at 4^0C. Preparation of samples prior to storage was as follows: fixation in FAA (70% ethanol: acetic acid: formalin (9:0.5:0.5)) for 18 to 48 hrs, followed by a 1 hr soak in 70% ethanol, samples were then stored in 80% ethanol up to 2 weeks.

6.1.2.5 Preparation of samples for confocal microscopy

Preliminary staining with fast green: 5 - 10 somatic embryos, placed in universal jars, were stained with fast green (15 ml methyl cellosolve, 15 ml clove oil, 0.2-0.3 g fast green, 90 ml 95% ethanol and 30 ml glacial acetic acid) for 5-15 min depending on the size of samples.

Fast green staining under vacuum: 5 - 10 embryos, in universal jars, were stained in fast green under vacuum for 4 hr and overnight.

Staining of nuclei with propidium iodide (Running *et al.* 1995): 5-10 embryos, in universal jars, were fixed overnight in FAA at room temperature. Samples were brought through an ethanol series (70, 85, 95 and 100%) with 30 min or more between each ethanol change and left in 100% ethanol overnight. Staining was performed by bringing samples through a decreasing ethanol series (95, 85, 70, 50, 30 and 15%) to distilled water, with 30 min or more at each change. Samples were stained in 1 ml of PI solution (5 μg/ml PI in a solution of 0.1 M L-arginine(HCl), pH 12.4) kept in the dark at 4^0C for 4 days. Samples were rinsed with 0.1 M L-arginine(HCl) buffer, pH 8 (adjusted with HCl or NaOH) at 4^0C for 4 days without agitation. The rinse solution was changed once per day. Clearing of samples was achieved by bringing samples through an ethanol series (15, 30, 50, 70, 85, 95, 100, 100, and 100), with 30 min or more per change. Samples were then brought through a xylene series (ethanol:xylene - 75:25, 50:50, 25:75, 0:100, 0:100 and 0:100), with a minimum of 2 hr between changes.

6.1.2.6 Viscosity of culture medium

After 4 weeks growth of calli on embryo induction medium, the viscosity of the cell free culture medium was determined using a Bolin Vor Rheometer system (Bolin). Viscometer parameters were set as follows: measuring system C 14, Torque element 0.307 g cm, autozero time delay 3s, integration time 3s constant time delay 3s, temperature 25^{0}C and sensitivity 1x. Media for viscosity measurements were pooled from all replications for each treatment.

6.1.2.7 Embryo maturation and regeneration

For each cultivar, globular embryos were transferred directly to regeneration (Rg4) medium (MS + 0.2 g/l glutamine + 3% sucrose). 'Aspiring' embryogenic calli, initiated on 3.0 μM 2,4-D produced more globular embryos than other cultivars and PGR treatments. For this reason, these embryos were used in a maturation study. The following maturation media were used: M1 (MS + 10 % glucose), M2 (MS + 2% sucrose), M3 (same as Rg4) and M4 (MS + 10 μM ABA + 2% sucrose). All experimental conditions for maturation and regeneration were similar to those used in experiment 2 (Chapter 3).

6.1.2.8 RAPD analysis of calli

A DNA profile for each type of callus was developed for all cultivars at each PGR treatment, using RAPD techniques. All experimental conditions, PCR reactions and DNA extraction protocols were the same as used for experiment 4 (Chapter 5). Four primers (OPC-01, -02, -07 and -08) were selected and used because they revealed more DNA polymorphisms in previous studies than the other primers (OPC-09, -10, -11, -12, -13, and -14). DNA profiles generated for each calli type were compared with that of the original explant and between calli types.

6.1.2.9 Cytological analysis

Chromosome number and DNA content of calli were determined as described in experiment 6 (Chapter 5).

6.1.3 RESULTS

6.1.3.1 Callus phenotype

Eleven calli types were identified (Table 6.1). A colour chart for all colours referred to in this section is found in Appendix Plate 3.0. Several calli types were observed for each PGR treatment. Cultivars treated with 0.3 µM 2,4-D characteristically produced only one type of callus, the exception being 'TU' for which 3 different calli types were observed. A maximum of 4 calli types were produced for some PGR treatments: 'KP' (1.0 µM NAA/0.1 µM Kinetin), 'SN' (1.0 µM NAA/0.1 µM Kinetin and 3.0 µM 2,4-D) and 'UC' (10 µM). A summary of each callus type, the specific cultivar, and PGR treatment associated with them is shown in Table 6.1. Gross morphological characteristics of each callus type are shown in Plate 6.1. Several calli types (A, E, H, I and K) consisted of large easily visible organised nodular structures, while friable callus contained smaller nodular structures. These nodular structures were typically located on the surface of the callus mass, except for mucilaginous calli where they were surrounded by mucilage.

6.1.3.2 Characteristic of cell suspensions from each calli type

The embryogenic potential of calli transferred to PGR-free embryo induction (EI) media was determined by the presence of globular embryos in suspension (Table 6.2). Globular embryos were produced for those calli types containing nodular structures. Cell suspensions consisted of one of the following:

- a heterogeneous population of globular embryos of varying sizes and single cells in suspension;
- masses of calli with no cells in suspension;

- masses of calli with embryogenic cells and mature embryos in suspension;
- mature embryos with root development; and,
- masses of calli with evidence of rhizogenesis (Table 6.2).

The appearance of the culture media also varied with cultivar and PGR treatment. After 4 weeks growth of calli in EI media, the culture media was either clear ('c') with no cells in suspension, turbid ('t') with cells in suspension or viscous ('v'). Viscous culture media were characteristic of calli types A and E initiated and maintained on PGR treatments 3.0 and 10 μM 2,4-D and were typical of cultivars ASP, KP, PC and UC. These mucilaginous calli were associated with high embryogenic potential (Table 6.2). The viscosity of the culture media after 4 weeks growth of different calli types is shown in Fig 6.1. Turbid culture media were not produced for any specific cultivar or PGR treatment, and were observed for the following calli: 'ASP' (0.3 μM 2,4-D, callus type J; 1.0 μM 2,4-D, callus type B and 10 μM, callus type C, H & J); 'KP' (1.0 μM 2,4-D, callus type F); 'PC' (3.0 and 10 μM 2,4-D, callus types A, E & K and 1.0 μM NAA/ 0.1 μM Kinetin, callus type J); 'TU' (3.0 μM 2,4-D, callus type E; 1.0 μM NAA/ 0.1 μM Kinetin, callus type I) and 'UC' (1.0 μM 2,4-D, callus type G). Light microscopic examination of the culture media, 4 weeks after callus growth, revealed that embryogenic cells and early stage globular embryos in suspension were present in those cultures classified as being viscous and or turbid. Differentiation of cells observed in embryogenic suspension cultures is shown in Plate 6.2. Three morphologically distinct globular embryos were detected. Varying percentage of embryos containing chlorophyll (chl+) were detected for all cultivars except 'KP' (Table 6.3). About 70% of chl+ embryos were developed from calli maintained on 1.0, 3.0 and 10 μM 2,4-D. Chlorophyll deficient (chl-) embryos were produced for all cultivars under each PGR treatment. A few globular embryos contained purple pigmentation and were characteristic of 'ASP' embryogenic callus type B initiated on 1.0 μM 2,4-D and 'SN' calli H initiated on both 1.0 μM 2,4-D and 1.0 μM NAA/0.1 μM Kinetin (Table 6.3).

procedures. Confocal microscopic images of each type of globular embryo showed distinct cellular organisation and development of the root primordia (Plate 6.3a-c). Approximately one in 10 embryos (chl+ and chl-) had a clearly developed shoot meristem region (Plate 6.3d). For other embryos unstructured, undifferentiated cell development was observed at the shoot end (Plate 6.3a-b). While both rhizogenesis and recurrent embryogenesis were common for all callus types, no pattern in their occurrence was observed.

6.1.3.3 Embryo maturation and regeneration

More than 10% regeneration of globular and mature embryos was observed for 'ASP' and 'TU' only. Plantlets regenerated from these somatic embryos were derived from calli types A, E and H for 'ASP' and B and H for 'TU' (Table 6.4). Transfer of globular embryos from embryo induction onto maturation media M1, M2, M4 and Rg4 resulted in a 16 , 20, 8, and 29 % embryo conversion respectively (Table 6.5). Less than 1% regeneration of globular somatic embryos was observed for the other cultivars (Karapiro, Pacifica, Syn4, and UC157).

6.1.3.4 RAPD analysis of calli

More changes to DNA structure were observed for 'KP', followed by both 'ASP' and 'PC' and then 'SN', 'TU' and 'UC' (Table 6.6). When the total number of variant DNA profiles for all cultivars is considered, PGR treatments 3.0 and 10 μM 2,4-D induced most changes to genomic DNA structure. Variant DNA profiles, generated by different primers, were observed for all calli types except G and I. Of the two most embryogenic calli (types A and E) type E was more genetically stable with only 2 incidences of variant DNA profiles detected. These aberrant profiles were observed for 'ASP' and 'PC' calli grown on 3.0 and 10 μM 2,4-D respectively. Banding patterns generated by primer OPC-01 revealed more DNA variation than primers OPC-02, -07 and -08. Changes to DNA profile of the original explant were observed as either an addition or absence of DNA band(s) (Table 6.6). Primer OPC-07 revealed changes in DNA profile for all calli types produced for cultivar KP, these changes were however mainly due to varying DNA band intensities.

for all calli types produced for cultivar KP, these changes were however mainly due to varying DNA band intensities.

6.1.3.5 Cytological analysis of calli

The chromosome number of calli were remained the same as the explant (2n = 20) and was not influenced by calli type. The DNA content of samples was diploid.

Table 6.1 Calli types developed for asparagus cultivars [Aspiring (ASP), Karapiro (KP), Pacifica (PC), Syn4 (SN), Turoa (TU) and UC157 (UC)] after 1½ years growth on callus induction medium. Calli characteristic for a specific cultivar and treatment are shown. Numbers 1-5 in the table represents plant growth regulator treatments of 0.3, 1.0, 3.0 and 10 μM 2,4-D and 1.0 μM NAA/0.1 μM Kinetin respectively.

Callus types	Description of callus	Cultivars with callus type	Treatment producing callus type
A	yellow, mucilaginous, nodular	KP, PC, UC	3, 4
B	tan friable	ASP, TU	1, 2
C	white crystalline	ASP, KP, PC, SN, TU	All
D	yellow compact	KP, PC, SN, UC	1, 2, 5
E	semi-mucilaginous, tan nodular	KP, PC, TU, UC	3, 4
F	yellow loose	KP, SN, TU	2, 3, 5
G	tan with embryos present	SN, UC	2, 3, 5
H	yellow nodular	ASP, SN, TU, UC	All
I	organogenic, nodular	ASP, TU	5
J	yellow compact with root extensions	ASP, KP, PC, UC	1, 4, 5
K	semi-mucilaginous yellow nodular	KP, PC, SN	3, 4

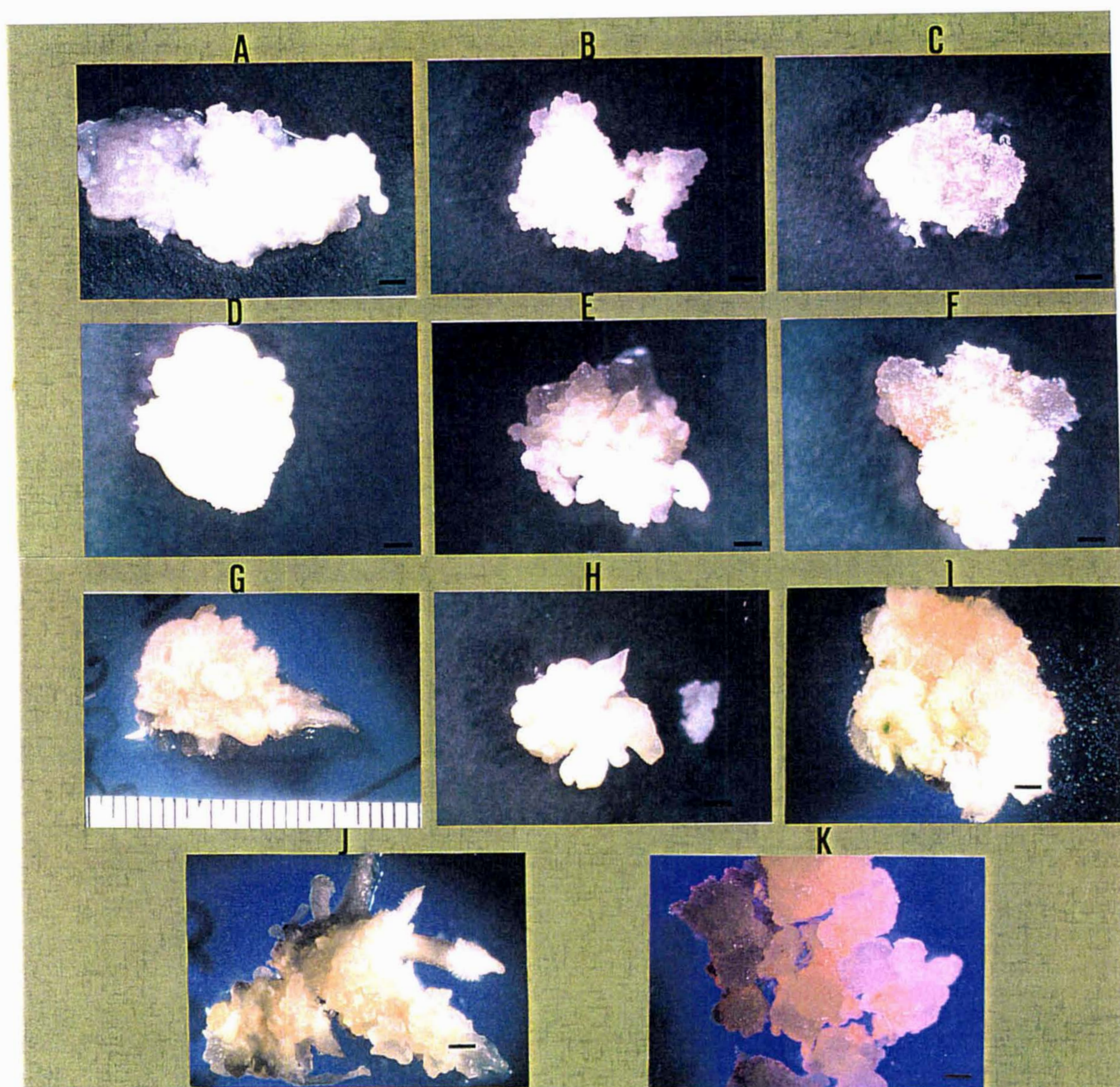

Plate 6.1 Phenological characteristics of calli types.

A Yellow, mucilaginous, nodular (type A), Bar = 0.5 mm; B Tan friable callus (type B), Bar = 0.5 mm; C White crystalline (type C), Bar = 0.5 mm; D Yellow compact (type D), Bar = 0.9 mm; E Semi-mucilaginous, tan nodular (type E), Bar = 0.5 mm; F Yellow loose (type F), Bar = 0.5 mm; G Tan callus with embryos (type G), Bar = 0.5 mm; H Yellow nodular (type H), Bar = 0.6 mm; I Organogenic, nodular (type I), Bar = 0.9 mm; J Yellow, compact callus with root extensions (type J), Bar = 0.6 mm; and K Semi-mucilaginous, yellow nodular (type K), Bar = 0.9 mm.

Table 6. 2 Types of calli developed for asparagus cultivars Aspiring, Karapiro, Pacifica, Syn4, Turoa, and UC157 maintained on PGR medium and the type of cell suspension produced when calli are transferred to PGR-free media. Numbers 1-5 in the table represents plant growth regulator treatments of 0.3, 1.0, 3.0 and 10 µM 2,4-D and 1.0 µM NAA/0.1 µM Kinetin respectively. Key to table symbols: 'A' - yellow, mucilaginous, nodular; 'B' - tan friable, 'C' - white crystalline, 'D' - yellow compact, 'E' - semi-mucilaginous, tan nodular, 'F' - yellow loose, 'G' - tan with embryos present, 'H' - yellow nodular, 'I' - organogenic, nodular, 'J' - yellow compact with root extensions and 'K' - semi-mucilaginous yellow nodular. 'c', 't' and 'v' in the table indicates culture media that were clear, turbid or viscous after 3 weeks calli growth on embryo induction media. 'ME' - mature embryo and 'EM' - embryogenic mass.

Cultivar	Treatment	Callus phenotype	Appearance of media	Embryogenic potential	Type of suspension
Aspiring	1	J	t	+	Callus clumps
	2	B	t	++	Heterogeneous
	3	A	v	+++	Heterogeneous
	4	C	t	-	Callus clumps
		H	t	+	Rhizogenesis
		J	t	++	Heterogeneous
	5	C	c	-	Callus clumps
		I	c		Callus clumps
Karapiro	1	D	c	-	Callus clumps
	2	D	c	-	Callus clumps
		F	t	+	callus clumps and ME
	3	A	c	+	ME with root extensions
		K	c	+	EM with root extensions
	4	A	v	+	EM with root extensions
		E	v	+	EM with root extensions
		K	c	-	Callus clumps
	5	C	c	-	Callus clumps
		D	c	-	Callus clumps
		F	c	+	callus clumps & root extension
		J	c	-	callus clumps
Pacifica	1	E	c	-	ME with root extensions
	2	C	c	-	Callus clumps
		D	c	-	Callus clumps
	3	A	v/t	+++	Rhizogenesis
		E	t	+++	Heterogeneous
		K	t	-	Suspension with root extension
	4	A	v/t	+++	Rhizogenesis
		E	t	++	Heterogeneous
		K	t	-	Suspension with root extension
	5	C	c	+	EM with root extensions
		D	c	+	suspension with root extension
		J	t	+	EM with root extensions

Table 6. 2 (continued) Types of calli developed for asparagus cultivars Aspiring, Karapiro, Pacifica, Syn4, Turoa, and UC157 maintained on PGR medium and the type of cell suspension produced when calli are transferred to PGR-free media. Numbers 1-5 in the table represents plant growth regulator treatments of 0.3, 1.0, 3.0 and 10 μM 2,4-D and 1.0 μM NAA/0.1 μM Kinetin respectively. Key to table symbols: 'A' - yellow, mucilaginous, nodular; 'B' - tan friable, 'C' - white crystalline, 'D' - yellow compact, 'E' - semi-mucilaginous, tan nodular, 'F' - yellow loose, 'G' - tan with embryos present, 'H' - yellow nodular, 'I' – organogenic, nodular, 'J' - yellow compact with root extensions and 'K' - semi-mucilaginous yellow nodular. 'c', 't' and 'v' in the table indicates culture media that were clear, turbid or viscous after 3 weeks calli growth on embryo induction media. 'ME' - mature embryo and 'EM' - embryogenic mass.

Cultivar	Treatment	Callus phenotype	Appearance of media	Embryogenic potential	Type of suspension
Syn4	1	H	c	+	Suspension with root extension
	2	H	c	+++	Heterogeneous
	3	C	c	-	Callus clumps
		F	c	-	Callus clumps
		G	c	+	Suspension with root extension
		H	c	-	Callus clumps
	4	K	c	++	Heterogeneous
	5	C	c	-	Callus clumps
		D	c	-	Callus clumps
		G	c	+	Suspension with root extension
		H	c	+++	Heterogeneous
Turoa	1	B	c	++	Heterogeneous
		C	c	-	Callus clumps
		H	c	+	Heterogeneous
	2	B	c	++	Globular
		F	c	-	Callus clumps
	3	E	t	++	EM with root extensions
	4	E	c	+++	Globular
		H	c	+	Suspension with root extension
	5	H	c	+	Heterogeneous
		I	t	+	Heterogeneous & organogenesis
UC157	1	J	c	++	Suspension with root extension
	2	G	t	+	Suspension with Globular
		H	c	+	Suspension with root extension
	3	A	v	+++	EM with root extensions
		E	c	+++	EM with root extensions
		H	c	+	Suspension with root extension
	4	A	v	+++	Heterogeneous
		E	v	+++	Heterogeneous
		H	c	-	Callus clump
		J	c	++	Suspension with roots
	5	D	c	++	Suspension with roots

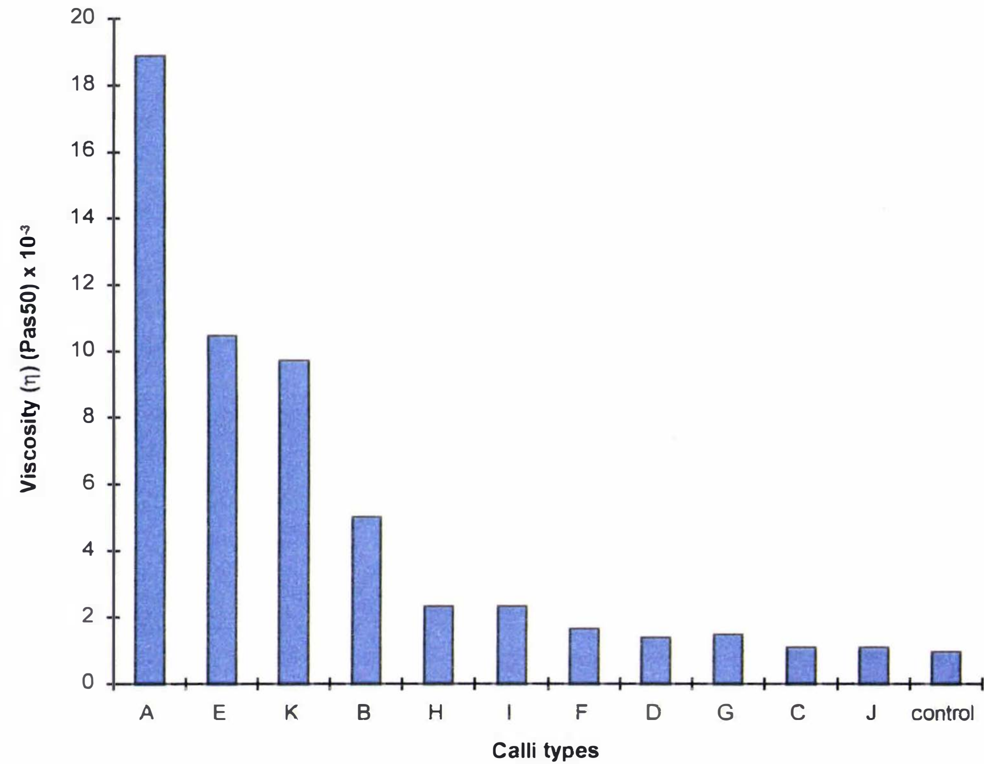

Figure 6.1 Viscosity of culture media 4 weeks after calli were transferred to PGR-free liquid embryo induction media (MS + 6% sucrose). Media from all replicates (n = 4) for each treatment were pooled.

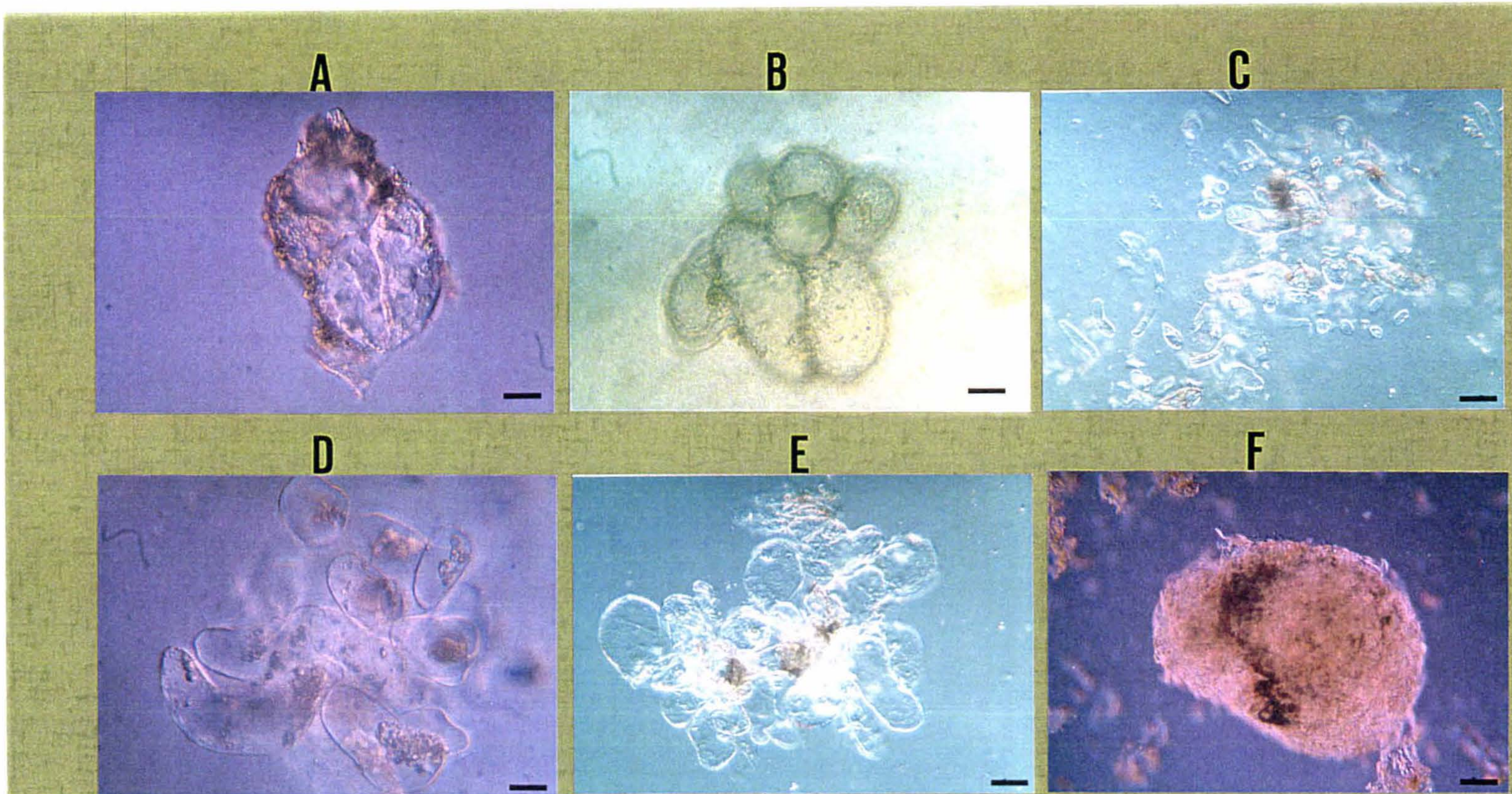

Plate 6.2 Cell differentiation in somatic embryo suspension cultures.

Dividing cells (A), Bar = 20 µm; Cell aggregate(B), Bar = 20 µm; Large vacuolated cells and smaller cells (C), Bar = 80 µm; Large vacuolated cells aggregating in a cluster (D), Bar = 20 µm; Cells aggregating (E), Bar = 20 µm; Early stage globular embryo (undifferentiated aggregate of densely cytoplasmic cells) (F), Bar = 80 µm.

Table 6. 3 Phenotype of globular embryos produced for asparagus cultivars after 4 weeks growth on embryo induction media with further development on regeneration (Rg4) media. Numbers 1-5 in the table represents plant growth regulator treatments of 0.3, 1.0, 3.0 and 10 μM 2,4-D and 1.0 μM NAA/0.1 μM Kinetin respectively. Key to table symbols: 'A' - yellow, mucilaginous, nodular; 'B' - tan friable, 'C' - white crystalline, 'E' - semi-mucilaginous, tan nodular, 'H' - yellow nodular, 'I' - organogenic, nodular, 'J' - yellow compact with root extensions and 'K' - semi-mucilaginous yellow nodular. 'c', 't', and 'v' in the table indicates culture media that were clear, turbid or viscous after 3 weeks calli growth on embryo induction medium. 'chl+' - globular embryos with chlorophyll, 'chl-' - chlorophyll deficient globular embryos and 'pur' - globular embryos with purple pigmentation.

Cultivar	Treatment	Callus type	Percent Globular embryos			Rhizogenesis	Recurrent embryogenesis
			chl+	chl-	pur		
Aspiring	1	A	0	100	0	+	-
	2	B	5	80	15	-	+
	3	A	0	100	0	+	-
	3	C	0	100	0	-	+
	3	E	90	10	0	-	-
	4	H	0	100	0	+	+
	4	K	0	100	0	+	-
Karapiro	4	A	0	100	0	+	+
	4	K	0	100	0	+	+
Pacifica	3	A	0	100	0	+	+
	4	A	0	100	0	+	-
	4	E	95	5	0	+	-
Turoa	1	B	5	95	0	-	+
	1	H	60	40	0	-	+
	3	E	5	95	0	-	+
	4	B	20	80	0	-	+
	5	H	10	90	0	-	+
	5	I	60	40	0	-	+
Syn4	2	H	30	50	20	-	+
	4	J	0	100	0	+	-
	5	H	20	70	10	+	+
UC157	3	A	0	100	0	+	-
	3	E	0	100	0	+	-
	4	A	0	100	0	-	+
	4	E	5	95	0	+	+

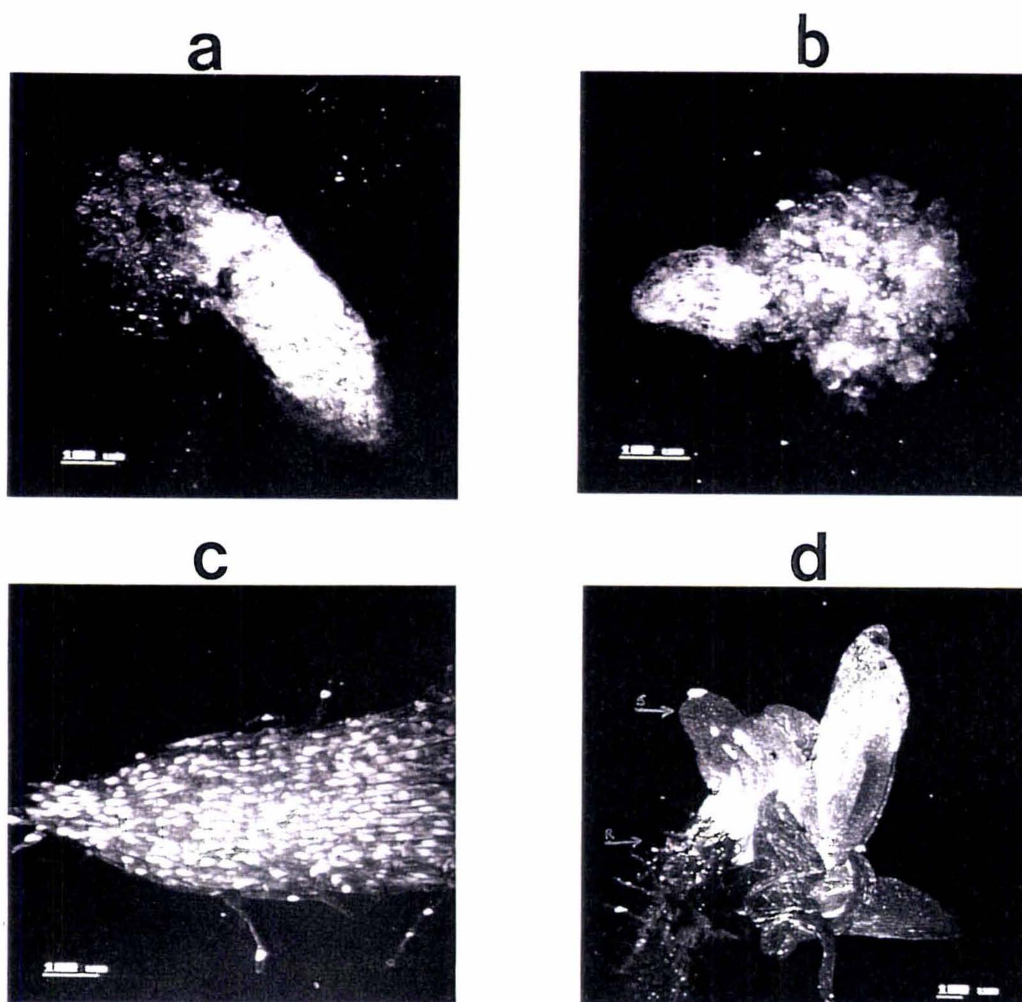

Plate 6.3 Confocal microscopic images of *Asparagus officinalis* L. somatic embryos

a-b Mature somatic embryos (stained with fast green under vacuum) with developed root system and undifferentiated cell development at the shoot end. Bar = 100 μm

c Root of mature somatic embryo (nuclei stained with propidium iodide). Bar = 100 μm

d Mature somatic embryo with both root and shoot development (embryos were stained with fast green under vacuum). Root with root (R→) and emerging shoot (S →) are indicated. Bar = 100 μm

Table 6. 4 Plantlets regenerated from cultivars Aspiring and Turoa globular and mature somatic embryos derived from various types of calli. Numbers 1, 3 & 4 in the table represents plant growth regulator treatments of 0.3, 3.0 and 10 μM 2,4-D respectively. Key to table symbols: 'A' - yellow, mucilaginous, nodular; 'B' - tan friable, 'E' - semi-mucilaginous, tan nodular and 'H' - yellow nodular. '*' includes plantlets from maturation study.

Cultivar	Treatment	Callus type	No. regenerated plantlets
Aspiring	1	A	5
	3	A	5
	3	E	73*
	4	H	9
Turoa	1	B	1
	1	C	4

Table 6. 5 Conversion of 'Aspiring' globular embryos derived from callus type E (semi-mucilaginous tan nodular) initiated on 3.0 μM 2,4-D, embryos were regenerated on Rg4 medium. chl+ - refers to globular embryos with chlorophyll.

Maturation media	No. somatic embryos	Type of embryo	No. of plantlets	% Conversion
M1	103	chl+	17	16
M2	56	chl+	11	20
M4	73	chl+	6	8
M3	127	chl+	37	29

M1 (MS + 10% glucose); M2 (MS + 2% sucrose); M3 (MS + 3% sucrose + 0.2 g/l glutamine); and M4 (MS + 10 μM ABA + 2 % sucrose).

Table 6. 6 Polymorphic DNA bands detected for calli maintained on callus induction media. '*' in the table indicates bands present in the original explant but which are absent in samples, '+' indicates presence of an intense band, '++' indicates presence of strong band, 'v' indicates variable bands observed for some PCR reactions and '-' indicates absence of a band. Numbers 1-5 represents plant growth regulator treatments of 0.3, 1.0, 3 & 10 µM 2,4-D and 1.0 µM NAA/0.1 µM Kinetin respectively. Calli types are represented by 'A' - yellow mucilaginous nodular, 'B' - tan friable, 'C' - white crystalline, 'D' - yellow compact, 'E' - semi-mucilaginous tan , nodular, 'F' - yellow loose, 'G' - tan with embryos, 'H' - yellow nodular, 'I' - organogenic, nodular, 'J' - yellow compact with root extensions, 'K' - semi-mucilaginous yellow nodular.

Polymorphic markers (bp)	Explant	Callus type and treatment							
Cultivar: Karapiro									
OPC01		3A	3K	4K					
-2000	-	v	++	++					
-700	-	-	++	++					
-650	-	v	-	-					
-600	-	+	-	-					
OPC02		1D	5D						
-600	-	++	++						
OPC07		1D	2F	3A	3K	4A	4K	5C	5D
-2000	v	+	+	+	+	+	+	+	+
-1400	v	+	+	+	+	+	+	+	+
-1300	+	+	+	+	+	+	+	+	+
-1200	v	++	++	++	++	++	++	++	++
-1000	-	v	v	v	v	v	v	v	v
-650	v	+	v	+	+	+	+	+	+
-600	+	*	*	*	*	*	*	*	*
-450	-	+	+	+	+	+	+	+	+
-300	-	+	+	+	+	+	+	+	+
-200	v	+	+	+	+	+	+	+	+

Table 6. 6 (continued) Polymorphic DNA bands detected for calli maintained on callus induction media. '*' in the table indicates bands present in the original explant but which are absent in samples, '+' indicates presence of an intense band, '++' indicates presence of strong band, 'v' indicates variable bands observed for some PCR reactions and '-' indicates absence of a band. Numbers 1-5 represents plant growth regulator treatments of 0.3, 1.0, 3 & 10 μM 2,4-D and 1.0 μM NAA/0.1 μM Kinetin respectively. Calli types are represented by 'A' - yellow mucilaginous nodular, 'B' - tan friable, 'C' - white crystalline, 'D' - yellow compact, 'E' - semi-mucilaginous tan , nodular, 'F' - yellow loose, 'G' - tan with embryos , 'H' - yellow nodular, 'I' - organogenic, nodular, 'J' - yellow compact with root extensions, 'K' - semi-mucilaginous yellow nodular.

Polymorphic markers (bp)	Explant	Callus type and plant growth regulator treatment			
Cultivar Aspiring					
OPC01		2H	3B	3E	4H
-1900	v	++	*	*	*
-1600	++	*	*	*	*
-1200	-	+	-	-	-
-1100	+	+	*	*	*
-1000	+	*	*	*	*
-750	-	+	-	-	-
-200	-	v	-	-	-
-150	++	v	*	*	++
-100	+	v	*	*	*
OPC02		5C			
-450	-	v			
-200	-	v			
Cultivar Pacifica					
OPC01		1J	4A	4E	4J
-1800	++	*	v	+	+
-1700	++	*	v	++	++
-700	-	v	+	-	-
OPC07		4J			
-1400	++	+			

Table 6. 6 (continued) Polymorphic DNA bands detected for calli maintained on callus induction media. '*' in the table indicates bands present in the original explant but which are absent in samples, '+' indicates presence of an intense band, '++' indicates presence of strong band, 'v' indicates variable bands observed for some PCR reactions and '-' indicates absence of a band. Numbers 1-5 represents plant growth regulator treatments of 0.3, 1.0, 3 & 10 µM 2,4-D and 1.0 µM NAA/0.1 µM Kinetin respectively. Calli types are represented by 'A' - yellow mucilaginous nodular, 'B' - tan friable, 'C' - white crystalline, 'D' - yellow compact, 'E' - semi-mucilaginous tan , nodular, 'F' - yellow loose, 'G' - tan with embryos , 'H' - yellow nodular, 'I' - organogenic, nodular, 'J' - yellow compact with root extensions, 'K' - semi-mucilaginous yellow nodular.

Polymorphic markers (bp)	Explant	Callus type and plant growth regulator treatment	
Cultivar Syn4			
OPC02		3H	5H
-500	v	++	++
Cultivar Turoa			
OPC01		2F	5B
-1700	-	v	-
-1500	-	-	+
-1200	-	+	+
-1000	++	+	*
-700	++	v	*
-500	-	++	-
-475	-	+	-
-400	-	+	-

Cultivar UC157

OPC01	UC_5 explant	2H	UC_6 Explant	3A
-800	++	v	*	*
-550	-	+++	-	-
-325	-	v	-	-
-150	-	-	-	+++
-100	-	v	-	-

6.1.4 DISCUSSION

Eleven calli types were identified and examined for their embryogenic potential. Embryogenic potential of calli was influenced by genotype and PGR concentration. Normally more than one type of callus was detected for each cultivar and for each PGR treatment. These results contrast with those of the callus induction study (Chapter 3, experiment 1) where only one type of callus was identified for each PGR treatment. This contradiction can be explained by assuming that, in the explant, different types of cells divide and proliferate to produce callus. Over time, and after several subcultures, the resulting callus will contain a mixture of cells of different genetic composition and probably different epigenetic potential (George & Sherrington 1984). The different calli types produced could then be selected.

Nodular calli were normally embryogenic, and mucilaginous nodular calli were more embryogenic than other calli types. Lack of embryogenic potential was generally associated with white crystalline callus (callus type C). Microscopic observations revealed that organised structures typical of globular embryos were present in nodular callus and that the 'nodules' present were embryos at different stages of development. Although detailed comparison of the embryogenic potential of various calli types, produced during initiation of asparagus embryogenic calli, has not been directly reported, several reports have alluded to the efficiency of specific calli to produce embryos (Levi & Sink 1991a & b; Mukhopadhyay & Desjardins 1994). Levi & Sink (1991a) note that friable calli used in their study consisted of several 'discrete groups of small embryogenic cells' that later developed into globular embryos. These cells could be equivalent to the 'nodules' observed in friable and mucilaginous calli identified in the present study. This type of friable callus was also shown to have embryogenic potential in other plant species, for example, bean (*Vicia faba*) (Grant & Fuller 1968) and maize (Armstrong & Phillips 1988).

Development of globular embryos was detected on medium containing PGRs. However, development was arrested at the immature globular stage (embryo size < 3mm), with further embryo growth commencing only after transfer of embryos to PGR-free embryo

induction media. On this media, several embryos reached the mature embryo stage (4-7 mm) with distinct development of the root and shoot primordia. Globular embryo development was restricted to those calli that developed nodular structures prior to transfer to PGR-free embryo induction medium. These results highlight the significance of the callus induction stage in the development of embryogenic determined cells for the establishment and maintenance of embryogenic cultures.

Large-scale production of asparagus somatic embryos in bioreactors requires that the system be inoculated with embryogenic cells in suspensions. Each type of callus identified was demonstrated to have the potential to produce cells in suspension. Suspensions developed from nodular and mucilaginous calli were more embryogenic than those developed from other calli types. The ability to produce embryogenic cells in suspension was influenced by genotype and PGR treatment. Calli grown on media containing high PGR concentrations (3.0 and 10 μM 2,4-D) were more efficient at producing suspension cultures than those developed on media containing low PGR concentrations (0.3 μM 2,4-D). Plant cells normally adhere to each other making it difficult to obtain suspensions of single cells (George & Sherrington 1984). Plant growth regulators such as auxins increased the activity of enzymes that cause the middle lamella of plant cells to disintegrate, thus enhancing separation of cells in suspension (George & Sherrington 1984).

Examination of globular embryos by confocal microscopy revealed that, while all mature embryos have well developed root primordia, only approximately 10% of them had a developed shoot meristem. About 90% of mature embryos examined developed a mass of undifferentiated cells around the shoot primordia. These results could explain the low percent conversion of globular embryos. It appears that only embryos with both root and shoot primordia develop into plantlets. In an earlier study (Chapter 3, experiment 2) it was observed that embryo shoot development occurred approximately 35 days after transfer to regeneration media. However, root development was observed about 10 days after mature embryos were transferred to regeneration media. Cladophyll development on some mature embryos were observed to occur 3 months after root growth. Embryo roots grew to about 5 to 10 mm before further growth stopped, and

only commenced growth after shoots developed. These results suggest that, while both root and shoot primordia are present on asparagus globular embryos, simultaneous development of these two primordia is not a frequent occurrence. More detailed anatomical studies, examining the development of somatic embryos over time, are required to determine precisely when or how both the root and shoot primordia develop in somatic embryos. Studies are also required to determine the effect a dormancy period, possibly induced by desiccation, has on development of shoot meristems.

Random amplified polymorphic DNA analysis of calli samples revealed that variant DNA profiles were more associated with the high PGR concentrations 3.0 and 10 µM 2,4-D than other treatments. These results were consistent with an earlier study conducted on asparagus embryogenic calli maintained on callus induction media for 1 year (Chapter 5, experiment 5). Of the two most embryogenic calli types, callus type A (yellow, mucilaginous and nodular) was more prone to mutations than callus type E (semi-mucilaginous, tan and nodular). It is interesting to note that calli types A and E, developed on callus induction media containing 3.0 and 10 µM 2,4-D, were more embryogenic than other callus. Unfortunately, more polymorphisms were revealed for these two calli types than for calli maintained on callus induction media containing low PGR concentrations (0.3 µM 2,4-D). Although it is interesting to note the degree of variation occurring during culture of embryogenic cells, it is more important to determine the percent of variation occurring in plantlets regenerated from somatic embryos. RAPD analysis of regenerated plantlets would give an indication of how much of the variation detected during culture is evident in plantlets. DNA analysis of plantlets recovered during this study is not yet possible. However, in a previous study (Chapter 5, experiment 5), RAPD analysis of 50 plantlets regenerated from somatic embryos did not reveal any changes in DNA structure from that of the original explant. These results indicate that variant cells detected in culture normally either did not survive or did not produce mature embryos that converted to plantlets. Therefore, growers can have confidence that a high proportion of plants produced by somatic embryogenesis will be true to type.

Primer OPC-01 revealed more variant DNA profiles than the other primers (OPC-02, -07, -08). This observation was consistent with that made during experiment 5 and

suggests that primer OPC-01 most probably amplifies regions within the genome that are more prone to variation. Cytological analysis was shown to be less sensitive than RAPD analysis in detecting changes to the asparagus genome.

6.1.5 SUMMARY

Of the 11 calli types identified during this study, only those calli with nodular structures produced embryogenic calli and globular embryos. Plantlets were regenerated for cultivars Aspiring and Turoa, and only from embryos derived from mucilaginous nodular calli. The embryogenic potential of different calli was influenced by plant genotype and PGR concentration. Simultaneous development of the root and shoot primordia did not occur in all globular embryos. Confocal microscopic images showed that 1 month after transfer to regeneration media, the root primordia developed in all embryos and that the shoot meristem only developed in 10% of embryos. The absence, or slow development, of the shoot primordia could be associated with the low conversion rates noted and could be a result of embryos going into a period of dormancy.

RAPD analysis of calli revealed that more variant DNA profiles were revealed for calli maintained on media containing 3.0 and 10 μM 2,4-D than for other PGR treatments. In addition, variant DNA profiles were detected for the most embryogenic callus types (A and E), although callus type E was less prone to somaclonal variation. These results demonstrate that callus type E (semi-mucilaginous, tan and nodular) is more efficient at producing genetically stable globular embryos than other calli types. Results from RAPD analysis and cytological analysis showed that plantlets regenerated from somatic embryos were genetically stable, having unchanged DNA profiles and ploidy levels.

Further studies are required to determine the factors that affect the simultaneous development of the shoot and root primordia in mature somatic embryos.

6.1.6 ACKNOWLEDGEMENTS

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SECTION 5

CHAPTER 7

7.1 GENERAL DISCUSSION AND CONCLUSION

Somatic embryogenesis is reported for over 200 plant species from both monocotyledonous and dicotyledonous families, and encompasses both angiosperms and gymnosperms (Tisserat *et al.* 1979; Bornman 1993). Although reports of asparagus somatic embryogenesis were made as far back as the 1950s, commercial applicability has been limited by several factors including: embryo initiation, embryo conversion somaclonal variation. In addition, industry demand is limited by the high cost of clones produced by somatic embryogenesis.

The major objective of the studies described in this thesis was to assess the genetic stability of plantlets regenerated from somatic embryos. In addition, protocols for the production of somatic embryos from six commercial asparagus cultivars were developed.

In experiment 1, the potential of asparagus cultivars Aspiring, Karapiro, Pacifica, Syn4, Turoa, and UC157 to produce embryogenic calli was evaluated. Callus initiation on explants was influenced by genotype, PGR type and concentration, and the source of the explant (i.e. greenhouse or *in vitro*). Normally, explants from greenhouse grown stock plants produced callus more efficiently than those maintained *in vitro*. Embryogenic potential was primarily influenced by plant genotype and PGR concentration. In a later study (experiment 8), it was observed that PGR type and concentration were key factors influencing the type of calli and embryogenic suspension produced for each asparagus genotype. Calli type determined the efficiency of embryogenesis for each cultivar. While all calli types (except white crystalline calli) produced early stage globular embryos, only, friable calli with nodules and nodular mucilaginous calli produced mature somatic embryos capable of converting to plantlets.

The genotype specific response to PGR treatment observed here, and in other studies, (Ghosh & Sen 1992; Levi & Sink 1991b) makes it difficult to develop generic protocols for production of asparagus somatic embryos. It was determined that plant growth

regulator treatments: 0.3, 1.0, 3.0, and 10 µM 2,4-D and 1.0 µM NAA/ 0.1 µM Kinetin could be used to produce embryogenic calli, and globular embryos in suspension for all cultivars.

Plant growth regulator type and concentration also influenced the development of somatic embryos from embryogenic calli. This was an indirect effect (dependent on the type of cell line developed during embryogenic callus induction) as somatic embryo development occurred in PGR-free medium.

Somatic embryogenesis has the potential for large-scale bioreactor production to produce propagules for commercial use (Giles & Friesen 1994; Reuther 1996). This has not been realised todate, as low embryo conversion efficiencies continue to be a problem in somatic embryogenesis of asparagus and other plants. Although we recovered plantlets for each cultivar, embryo conversion frequencies were low (5-35%). One of the proposed advantages for using somatic embryogenesis to produce large numbers of clonal material is the simultaneous development of both the shoot and root primordia on mature embryos, eliminating the need for different media to promote both shoot and root growth. Anatomical studies of globular and mature somatic embryos showed that approximately 10% of all embryos examined had developed both a shoot and root primordia. Lack of, or delayed, shoot primordium development on somatic embryos could explain the low conversion rates observed in this and other studies (Bornman 1993). Finkelstein & Crouch (1987) suggest that poor development, or inactivity, of the shoot apical meristem may be due to incomplete embryo maturation. Maturation of somatic embryos is therefore an important stage in the development of somatic embryos. None of the maturation treatments used in our study were effective at promoting embryo maturation and conversion to plantlets.

Treatment of embryos to temperatures at or below 0^{0}C more effectively enhanced asparagus somatic embryo conversion than higher temperatures. One hypothesis for the effectiveness of cold stress, is that somatic embryo development maybe arrested at a specific stage and further development only commences after a dormancy breaking treatment. In this study, cold stress may have activated transcription of developmental genes while silencing expression of embryogenic genes. Other equally effective

treatments such as desiccation, exogenous supplies of ABA, proline, and high osmoticum (Gary & Purohit 1991) can also increase the frequency of somatic embryo conversion and reduce occurrence of precocious germination.

Experiment 3 showed that acclimatisation of plantlets with a developed minicrown containing 2-5 storage roots improved survival of plantlets when transferred to the greenhouse. Storage roots contain carbohydrate reserves which can be used by existing cladophylls as an energy source. Removal of all *in vitro*-developed cladophylls, down to crown level, immediately prior to acclimatisation also enhanced survival of plantlets. This effect was probably due to a number of factors including:

- removal of cladophylls could protect acclimatised plantlets from water stress,
- removal of cladophylls poorly adapted for survival that could utilise storage reserves, and
- conservation of energy reserves for production of acclimatised spears and cladophylls.

The genetic stability of embryogenic calli, suspension cells, and regenerated plantlets were also investigated to determine the ability of asparagus somatic embryogenesis to produce genetically stable plantlets. Random amplified polymorphic DNA analysis of embryogenic calli and suspension cells detected the occurrence of somaclonal variation in some samples. Calli developed on media supplemented with high PGR concentrations (3.0 and 10 µM 2,4-D) were more unstable genetically than calli maintained on lower PGR concentrations (0.3 and 1.0 µM 2,4-D). In addition, mucilaginous calli types were more genetically unstable than other calli types. The type of primer used influenced the efficiency of RAPD techniques to reveal polymorphisms.

Results indicate that the ability of RAPD techniques to reveal variation in genomic structure may be strongly influenced by the region amplified by specific primers. Primer OPC-01 for example, revealed more polymorphisms than other primers, this was attributed to the possibility that OPC-01 amplified hypervariable regions in the genome that were prone to mutations. One limitation of RAPD techniques is that variation in

longer primers, each amplifying different regions of the genome, would give a more comprehensive indication of genetic variation occurring within the genome. The frequency of somaclonal variation detected by RAPD techniques was also influenced by plant genotype. The genome of 'Pacifica' was more unstable than that of other cultivars, indicating that the genome was probably more susceptible to inter-cellular stress factors associated with changes in DNA structure.

Cytological techniques (Chromosome counts and DNA content determination) were unable to detect somaclonal variation in embryogenic calli and suspensions that was detected by RAPD analysis. Cytological analysis of regenerated plantlets showed that their chromosome number was unchanged from that of the explant. Collectively, the results from RAPD and cytological analysis indicate that genetically stable asparagus plantlets were regenerated via somatic embryogenesis. These results also indicate that there is a positive selection for the development of genetically stable somatic embryos from embryogenic cells.

Extracellular protein profiles were examined for their potential use to detect somaclonal variation occurring during culture of asparagus somatic embryos. Protein profiles were influenced by plant genotype, PGR concentration and type, and length of time of culture. While protein profiles proved useful to distinguish among asparagus cultivars, they were of limited use to detect somaclonal variation. One factor limiting the use of extracellular protein profiles to reveal changes in genome structure is that mutations in protein coding genes can go undetected by standard protein analysis techniques as the mutation may not cause significant changes in protein migration rate. In addition, extracellular protein production is influenced by epigenetic effects (Bailey 1983). By contrast DNA profiles are unaffected by epigenetic effects and only vary when there is a change to genome (nuclear or organelle). Molecular techniques used here are more powerful tools for detection of somaclonal variation than either cytological or biochemical studies.

In conclusion these studies show that genetically stable plantlets were produced from asparagus ('Aspiring', 'Karapiro', 'Pacifica', 'Turoa', 'Syn4', and 'UC157') somatic embryos developed on medium supplemented with 2,4-D concentrations ranging from 0.3 - 10 µM. However, the process was limited by the ability of embryos to convert to complete functional plantlets.

7.2 APPLICATION OF THIS RESEARCH

Some of the results obtained, and protocols developed, during the research project can be directly applied to enhance commercial production of asparagus clones. Data obtained from microscopic analyses shows the potential for image analysis to be included in automated systems for the selection of somatic embryos with both root and shoot primordia.

7.2.1 Direct application

- Low temperatures can be used to enhance embryo regeneration and should be used on other asparagus cultivars to evaluate its wide spread applicability.
- Improvement of somatic embryo development and conversion by transferring mature embryos from a PGR-free MS medium containing 6% sucrose to a regeneration medium containing 3% sucrose and 0.2g/l glutamine.
- Acclimatisation of plantlets with 3 to 5 *in vitro*-developed storage roots to enhance survival.
- Removal of *in vitro*-developed cladophylls down to crown level can markedly enhance survival of acclimatised plantlets.

7.2.2 Future application

Asparagus somatic embryos can potentially be used as synthetic seeds. Synthetic seeds (synseeds) are defined as somatic embryos manipulated to be used for large scale plant production (Redenbaugh 1993). Development of synthetic seeds involves a series of steps that culminate in the establishment of a viable mature unipolar somatic embryo in a selected growing medium.

The following diagram (Fig 7.1) is a proposal for the production of asparagus synthetic seeds.

Production of Asparagus Synthetic Seed

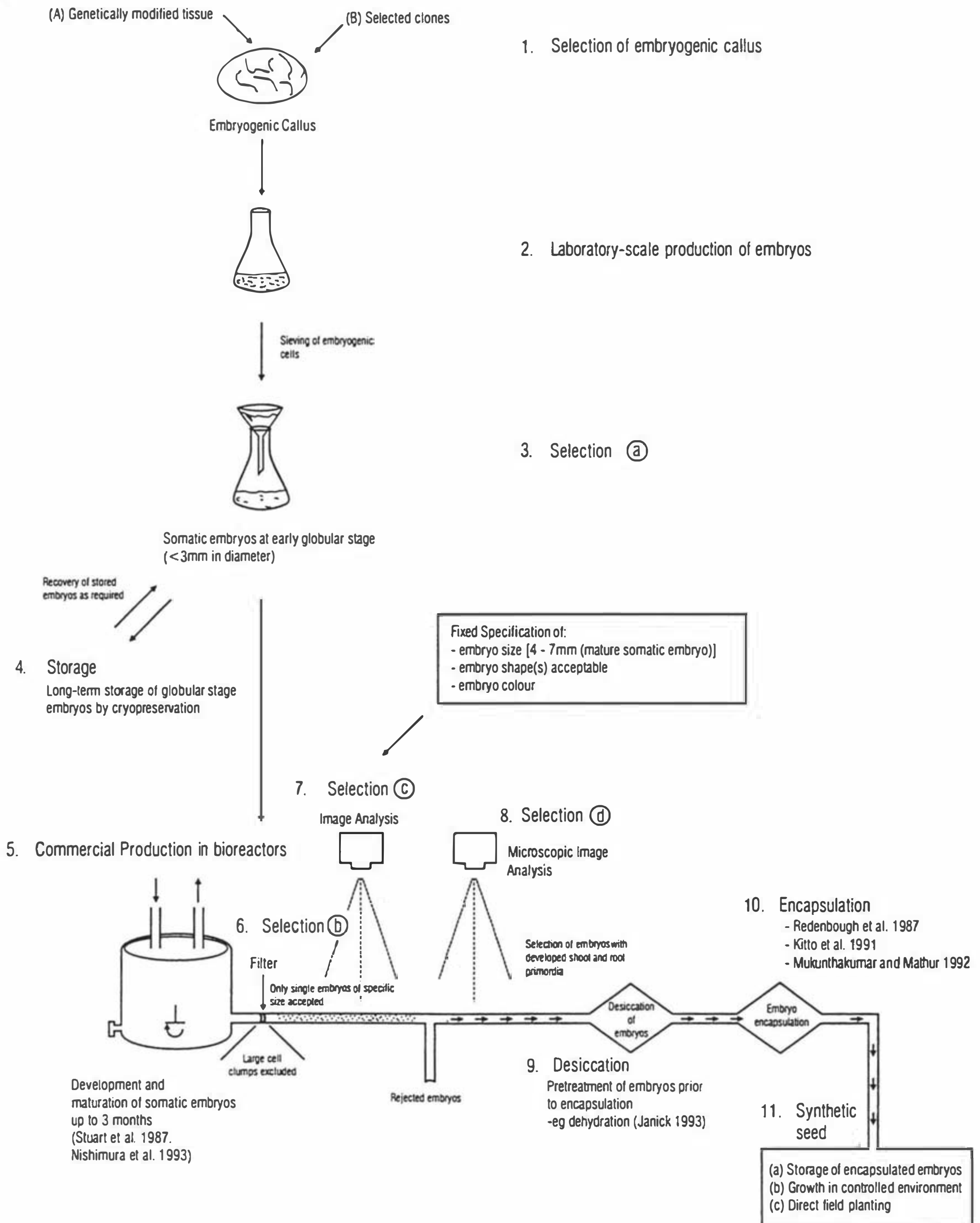

Figure 7.1 Schematic presentation of a hypothetical asparagus synthetic seed production system.

7.3 RECOMMENDATIONS FOR FUTURE RESEARCH

Several areas of asparagus somatic embryogenesis need further development to enhance embryo production and regeneration. The following sections highlights some of these areas.

7.3.1 Embryo maturation

Only 5 to 35 percent of globular asparagus somatic embryos produced viable plantlets. This low rate of embryo conversion limits the commercial applicability of asparagus somatic embryogenesis. For this reason, further research is required to develop effective maturation treatments to improve embryo conversion frequencies. These areas of research can include:

- Low temperature treatments, between -5^{0}C and 5^{0}C, for longer periods than used in our study. These treatments could either initiate embryo dormancy, allowing embryos to mature and accumulate energy reserved, or they could be used to break a period of voluntary embryo quiescence. The precise effect (i.e. dormancy initiating or breaking) of these treatments must be determined. In addition, the effect of low temperatures on the development of the shoot meristem should also be determined.
- Desiccation of somatic embryos to impose a period of dormancy on embryos. These treatments could enhance embryo maturation and the accumulation of storage reserves (Finkelstein & Crouch 1986, 1987; McKersie & Van Acker 1994).
- Determination of the biochemical and molecular factors that influence the responsiveness of bipolar somatic embryos to regeneration treatments.
- Anatomical analysis of developing somatic embryos to determine when shoot primordia develop on embryos, their development in relation to root meristem development, and their development in response to effective maturation treatments.

7.3.2 Genetic stability

The production of genetically identical plantlets is highly desirable for commercial propagation of asparagus clones via somatic embryogenesis. The above experiments used molecular techniques (RAPDs) and cytological analysis (chromosome counts and DNA content measurements) to show that plantlets regenerated form somatic embryos were genetically stable. The results could be confirmed by further analysing samples with other molecular techniques such as AFLPs, CFLPs and SSCPs which may be more sensitive than RAPD techniques and could therefore, be more effective at detecting changes in genome structure.

7.3.3 Bioreactor production

As reduced labour costs will continue to be important in *in vitro* asparagus production, it is most likely that efforts to make somatic embryogenesis more efficient will continue. If bioreactor production is to be one of approaches used for commercial production, it will require further development especially in synchronisation of embryo development and automation of the system.

CHAPTER 8

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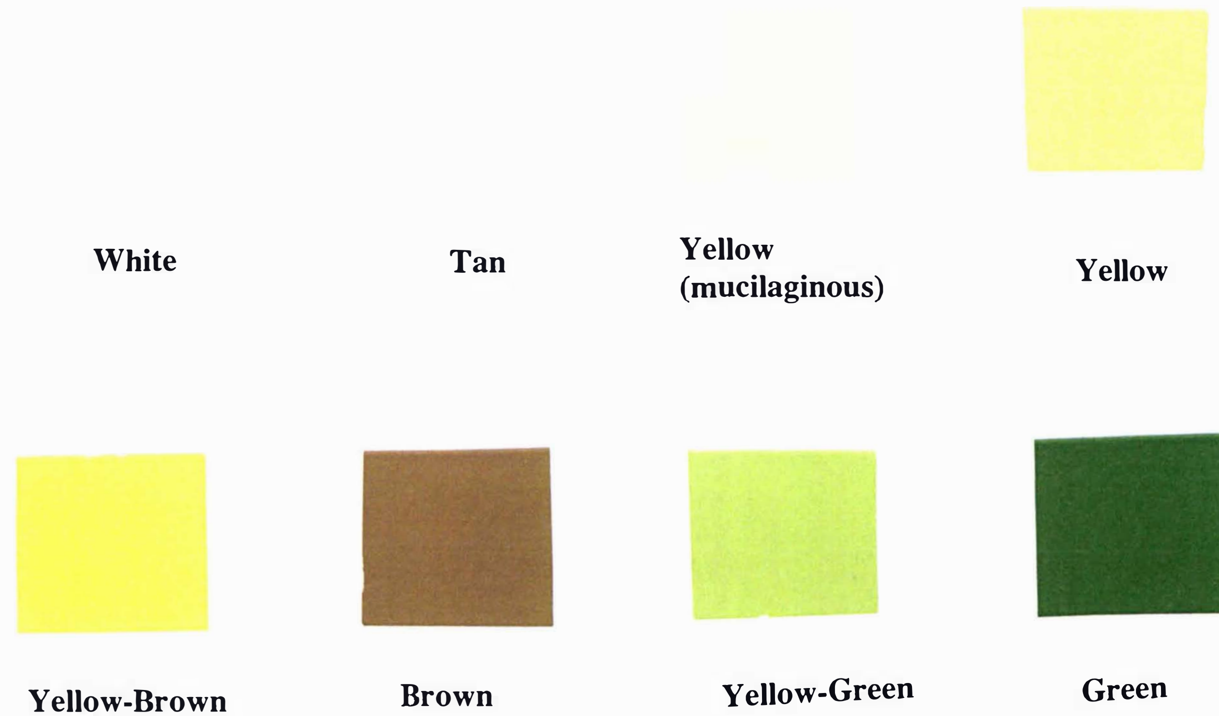

Plate 3.0 Colour chart. Sourced from Dulux® Roof paint, Timbacryl, and Paving paint colour charts (ICI Paints, New Zealand Ltd.).

Table 5.0 DNA profiles generated for asparagus (*Asparagus officinalis*) cultivars Aspiring (A), Karapiro (B), Pacifica (C), Syn4 (D), Turoa (E), and UC157 (F) using 20 10-base primers from Operon Kit C. Polymerase chain reaction (PCR) products were separated on 8% polyacrylamide gels and silver stained. Key to table symbols: 'v' = faint bands amplified for some PCR reactions; '+' = presence of intense bands amplified for all reactions but which vary in intensity; '++' = very strong bands present in all reactions with no variation in intensity. '0' = absence of a band. '*' = smear of bands.

Table 5.0 A 'Aspiring'

Polymorphic bands (bp)	Primers from Operon kit C																			
	C-01	C-02	C-03	C-04	C-05	C-06	C-07	C-08	C-09	C-10	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18	C-19	C-20
2000	0	++	0	0	0	v	0	0	0	0	0	0	0	0	++	0	0	0	0	++
1900	v	0	0	0	0	0	0	v	*	0	0	0	0	0	+	0	0	0	v	0
1800	0	v	v	0	0	0	0	0	*	v	0	0	0	0	+	0	v	0	v	0
1700	0	0	0	0	0	0	0	0	*	0	0	0	0	0	+	0	v	v	0	0
1600	++	v	+	++	+	++	0	0	*	0	+	0	0	+	0	0	v	v	0	0
1500	0	0	0	+	v	++	v	0	*	v	0	+	+	0	++	0	+	0	0	0
1400	0	v	v	+	v	0	0	+	*	v	0	0	++	v	+	0	0	0	+	0
1300	0	v	v	0	0	0	v	0	*	0	0	+	0	+	++	+	0	0	v	0
1200	0	0	0	+	0	0	0	++	++	0	0	v	0	0	0	0	0	0	+	+
1150	0	0	0	+	0	0	0	0	0	0	0	0	0	0	++	0	0	0	+	0
1100	+	+	0	0	0	v	0	0	++	0	0	++	0	0	0	0	0	0	0	+
1000	+	+	++	0	++	0	++	+	0	++	0	+	v	++	+	0	0	++	++	0
950	0	0	0	0	+	v	0	+	0	0	0	0	0	0	0	0	+	0	0	0
900	++	0	0	+	0	+	++	0	0	++	0	+	+	0	+	0	++	++	0	v
850	0	0	v	0	0	+	0	0	0	0	0	+	0	0	0	v	v	0	0	0
800	0	++	++	+	++	v	0	++	0	0	0	++	v	+	+	v	0	v	0	0
750	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	v	v	+
700	+	0	0	v	+	0	++	0	0	v	++	0	+	v	+	0	0	0	v	+
675	0	0	0	0	0	0	0	0	0	0	0	0	0	0	v	0	0	0	0	0
650	0	0	0	0	0	0	0	++	0	0	*	v	0	0	0	0	+	0	v	0
600	0	0	v	++	0	+	+	0	v	0	*	0	0	0	0	v	+	v	0	0
550	++	++	v	0	0	0	0	v	0	0	0	0	0	0	0	v	0	0	0	0
500	0	0	0	++	0	0	++	0	v	0	0	v	0	0	0	0	+	0	0	0
450	0	0	0	0	+	0	0	0	0	0	0	0	0	0	++	0	0	0	0	+
400	0	+	0	v	v	0	0	v	0	0	0	v	0	0	0	+	0	+	0	0
350	0	0	0	0	v	v	0	0	0	0	0	0	0	0	0	0	0	+	++	0
325	0	0	0	0	0	v	0	0	0	0	0	0	0	0	0	0	0	0	0	0
300	+	0	0	0	++	+	+	0	++	0	0	0	0	+	0	0	0	v	+	v
275	0	+	v	0	0	0	0	0	*	0	0	0	0	0	0	+	0	0	+	0
250	v	0	v	0	0	0	0	0	*	0	0	0	0	0	0	0	v	0	0	0
225	0	0	0	0	+	0	0	0	*	0	0	0	0	0	0	0	+	0	0	0
200	0	0	0	+	v	0	0	0	*	++	0	0	0	0	0	0	0	+	0	+
150	++	0	v	0	0	0	v	0	*	0	0	0	0	0	0	0	+	v	0	0
100	+	++	0	+	0	0	v	0	++	0	0	0	0	0	0	0	0	v	0	0

Table 5.0 B 'Karapiro'

	Primers from Operon kit C																			
Polymorphic bands (bp)	C-01	C-02	C-03	C-04	C-05	C-06	C-07	C-08	C-09	C-10	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18	C-19	C-20
2000	0	0	0	0	0	0	v	0	0	0	0	0	0	0	0	0	0	0	0	0
1900	+	+	v	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0
1800	0	0	v	0	0	0	0	0	*	0	0	0	0	0	0	++	0	0	0	0
1700	0	+	v	v	++	0	0	0	*	0	0	0	0	0	v	0	+	0	+	0
1600	0	0	0	0	0	0	0	+	*	0	+	v	++	+	+	0	+	0	+	0
1500	0	0	0	0	++	0	0	0	*	0	0	v	0	0	+	+	+	0	0	+
1400	0	0	v	+	0	v	v	0	*	0	0	v	0	v	0	v	+	0	++	0
1300	0	0	v	v	0	0	+	0	*	0	0	+	++	0	0	0	0	0	0	0
1200	0	0	0	0	0	v	v	+	++	0	0	+	+	++	0	0	0	0	0	0
1100	0	0	0	+	v	0	v	0	v	0	v	0	+	0	v	0	0	+	0	v
1000	0	v	0	0	v	++	0	0	v	0	v	++	0	v	0	++	+	v	0	+
950	0	0	+	0	0	0	+	0	0	+	0	0	0	0	0	0	0	0	++	0
900	v	v	0	++	++	++	v	0	0	++	0	+	0	+	v	0	v	0	0	v
850	0	0	0	0	0	v	0	0	0	0	0	+	0	+	0	0	0	0	v	0
800	v	0	0	0	++	I	+	0	v	0	0	+	0	0	0	0	v	0	0	+
750	0	0	0	+	0	0	0	0	0	0	0	0	++	0	0	v	0	0	v	0
700	0	v	0	0	0	+	0	0	v	v	0	0	+	0	v	v	0	0	++	v
650	0	+	0	v	0	++	0	0	0	0	0	v	0	v	0	+	0	0	v	0
600	0	0	0	v	++	0	+	0	++	v	++	0	0	0	v	+	0	0	+	0
550	0	0	++	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	v
500	++	++	0	++	+	0	0	0	0	0	0	0	+	0	v	+	0	v	+	v
450	0	0	0	v	++	0	0	0	0	0	0	0	0	0	+	0	0	0	0	+
400	+	0	0	0	0	0	v	++	++	0	0	+	+	0	0	+	0	+	+	0
350	0	0	0	v	++	0	0	0	*	0	0	0	0	0	0	0	0	+	0	v
325	0	0	v	0	0	0	0	0	*	0	0	0	0	0	0	0	0	0	0	0
300	0	v	0	0	0	0	0	++	*	0	0	0	0	0	0	0	0	+	0	v
275	0	0	0	+	0	0	0	0	*	0	0	0	0	0	0	0	0	0	v	0
250	0	0	+	0	0	0	+	0	*	0	0	0	0	0	0	0	0	0	0	++
225	+	0	0	0	0	0	0	0	*	++	0	0	0	0	0	0	0	+	+	0
200	+	0	0	0	0	+	v	0	*	++	0	0	0	0	0	0	0	0	0	++
150	0	v	0	0	0	+	0	0	++	0	0	0	0	0	0	0	0	0	0	
100	0	0	0	0	v	0	0	+	0	0	0	0	0	0	0	+	v	0	v	v

Table 5.0 C 'Pacifica'

Polymorphic bands (bp)	Primers from Operon kit C																			
	C-01	C-02	C-03	C-04	C-05	C-06	C-07	C-08	C-09	C-10	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18	C-19	C-20
2000	+	0	0	0	0	0	0	++	0	0	+	0	0	0	0	0	0	0	0	0
1900	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0
1800	++	++	0	v	0	0	0	0	0	0	0	0	+	v	0	++	0	++	0	+
1700	++	0	v	0	+	0	0	0	0	0	0	0	0	0	++	0	++	0	0	+
1600	0	v	0	+	0	0	0	0	+	v	v	0	0	+	++	0	0	0	0	0
1500	0	v	0	0	++	0	0	0	++	0	0	0	++	0	++	++	0	0	0	0
1400	0	0	0	+	+	+	++	0	*	0	0	+	0	+	0	0	0	+	0	0
1300	0	v	0	0	0	0	0	v	*	0	0	v	++	+	v	0	0	+	++	0
1200	v	0	0	0	+	0	++	0	*	+	+	0	0	++	v	0	0	0	0	v
1150	0	0	0	0	0	0	v	0	*	0	+	0	0	0	0	0	0	0	0	0
1100	0	v	v	0	0	++	0	0	*	0	0	++	+	v	0	++	0	0	+	v
1000	v	0	++	+	++	0	0	+	*	+	+	+	+	v	+	0	+	0	0	0
950	0	++	0	0	0	0	0	0	*	0	0	+	0	0	+	0	0	++	0	0
900	0	0	0	++	0	0	0	0	*	+	0	0	+	+	0	+	+	0	+	0
850	v	0	0	0	0	0	0	v	*	0	0	0	0	0	0	0	0	0	0	0
800	v	0	++	+	++	+	0	+	v	0	0	++	0	0	+	+	+	0	+	++
750	0	0	0	0	0	++	0	++	0	0	0	0	++	0	0	0	0	+	0	0
700	0	v	0	0	v	+	++	0	+	0	++	0	++	v	+	+	0	0	v	v
675	0	0	0	0	0	0	0	0	0	0	*	0	0	0	0	0	0	0	0	0
650	++	0	0	0	0	0	0	0	0	0	*	0	0	+	0	0	v	0	0	0
600	0	++	0	v	v	+	0	0	0	0	++	0	0	0	0	0	0	v	+	0
550	0	0	0	++	0	0	0	+	+	0	0	0	0	v	0	0	v	0	v	0
500	0	0	0	++	+	v	0	0	+	0	0	+	+	0	0	0	0	0	0	+
475	0	0	0	0	0	0	0	0	*	0	0	0	0	+	0	0	0	v	0	v
450	0	0	0	0	0	0	0	0	*	v	0	0	+	0	+	0	v	0	0	0
400	v	++	+	v	+	v	0	0	*	0	0	v	+	0	0	v	0	v	+	0
350	0	++	+	0	0	v	+	0	*	0	0	0	0	0	0	0	+	+	0	0
325	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0
300	0	0	0	v	+	v	0	0	++	0	0	0	0	0	0	0	0	++	0	+
275	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0
250	0	0	+	0	0	0	0	0	0	++	0	0	0	0	v	0	0	0	0	0
225	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
200	v	++	0	0	++	++	0	0	0	0	0	0	0	0	0	v	+	++	v	v
150	v	v	v	0	0	++	v	0	0	++	0	0	0	0	0	v	+	v	0	0
100	0	++	v	0	v	++	0	0	0	0	0	0	+	0	0	0	0	v	0	v

Table 5.0 D1 'Syn4_7'

Polymorphic bands (bp)	Primers from Operon kit C																			
	C-01	C-02	C-03	C-04	C-05	C-06	C-07	C-08	C-09	C-10	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18	C-19	C-20
2000	0	0	0	0	0	0	+	0	0	0	v	0	0	0	v	0	0	0	0	v
1900	0	++	0	0	0	v	0	+	0	0	0	+	0	0	0	0	v	0	+	0
1800	+	0	0	0	++	0	0	+	*	0	0	+	0	+	+	+	v	0	++	0
1700	0	++	0	+	0	v	0	+	*	0	+	+	0	0	++	v	+	0	0	+
1600	0	0	0	0	0	0	+	0	*	0	0	+	+	v	++	v	+	0	0	+
1500	0	+	0	+	0	v	+	0	*	0	0	+	0	0	0	v	+	0	0	0
1400	0	0	v	0	0	0	+	0	*	0	v	+	0	0	0	0	+	0	0	0
1300	0	+	0	++	++	+	++	0	*	0	v	+	0	0	0	++	v	0	0	0
1200	0	+	0	+	v	++	0	0	*	0	0	v	++	v	0	+	v	0	v	0
1150	0	+	0	+	0	0	0	0	++	0	0	0	0	0	0	0	++	0	v	0
1100	0	+	+	+	v	+	+	+	+	0	0	++	0	0	v	0	0	0	+	v
1000	v	0	0	v	0	+	0	+	+	0	++	++	0	++	++	0	0	+	+	v
950	0	0	0	0	0	0	0	+	0	0	0	0	0	0	*	0	0	++	v	+
900	v	++	0	+	++	++	+	++	v	+	0	++	++	++	*	0	v	0	0	0
850	0	+	0	0	0	0	+	0	0	0	0	0	0	0	*	0	v	0	0	+
800	++	0	0	+	v	0	+	v	0	0	++	++	v	0	++	0	+	0	0	0
750	0	0	+	0	0	+	0	0	0	0	0	0	0	0	+	0	0	+	0	0
700	+	v	0	++	0	0	0	0	0	++	v	0	v	v	0	0	0	+	0	+
675	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	+	0	0	0	+
650	0	0	0	++	0	0	0	0	0	0	+	0	0	0	0	v	0	++	0	+
600	0	0	0	0	+	0	++	v	0	0	+	0	+	0	v	0	+	0	0	0
550	0	0	0	++	++	0	0	0	0	0	0	0	+	0	+	0	+	0	0	0
500	++	v	0	0	+	0	0	0	0	v	0	0	0	v	0	0	0	0	0	0
450	0	++	+	++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
400	++	++	0	0	0	+	0	++	0	0	0	0	+	0	0	v	0	0	+	0
350	0	0	0	0	0	0	0	*	++	0	0	v	0	0	v	0	+	0	0	++
325	0	0	v	0	0	0	v	*	0	0	0	0	0	0	+	0	0	0	++	0
300	0	++	0	0	0	0	v	*	0	0	+	0	0	v	++	0	0	0	0	0
275	v	0	0	0	0	0	0	*	0	0	0	0	0	0	0	0	0	0	0	0
250	0	v	0	0	0	0	v	*	0	0	0	0	0	0	0	0	v	0	0	+
225	v	0	+	0	0	0	0	*	+	0	0	v	0	0	0	0	v	0	v	0
200	0	++	0	0	0	0	v	++	0	0	+	0	+	0	0	0	0	0	v	0
150	0	0	0	0	0	0	0	0	+	0	v	0	0	0	++	0	0	0	0	0
100	+	++	v	0	0	0	0	0	+	++	v	0	+	v	0	0	v	+	0	+

Table 5.0 D2 'Syn4_13'

Polymorphic bands (bp)	Primers from Operon kit C																			
	C-01	C-02	C-03	C-04	C-05	C-06	C-07	C-08	C-09	C-10	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18	C-19	C-20
2000	0	0	0	0	0	0	0	0	*	0	0	0	0	0	v	0	0	0	0	v
1900	0	0	0	0	0	v	0	0	*	0	0	0	0	0	0	0	v	0	+	0
1800	+	+	0	0	++	0	0	0	*	0	0	0	0	+	+	+	v	0	++	0
1700	0	0	0	+	0	v	0	v	*	0	0	0	0	0	++	v	+	v	0	+
1600	0	0	0	0	0	0	0	0	*	0	0	0	v	0	++	v	+	v	0	+
1500	0	0	0	+	0	v	+	0	*	0	0	v	0	0	0	v	+	+	0	0
1400	0	0	v	0	0	0	0	+	*	0	0	0	0	0	0	0	+	0	0	0
1300	0	0	0	++	++	+	+	+	*	0	0	+	0	+	0	++	v	0	0	v
1200	0	0	0	+	v	++	0	0	*	0	0	+	0	++	0	+	v	0	v	0
1150	0	0	0	+	0	0	0	0	*	0	0	0	+	0	0	0	++	0	v	0
1100	0	0	+	+	v	+	0	0	++	0	0	+	+	++	v	0	0	0	+	v
1000	v	v	0	v	0	+	0	+	0	0	0	0	0	++	++	0	0	+	+	v
950	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	++	v	+
900	v	v	0	+	++	++	++	++	0	0	0	+	0	v	+	0	v	0	0	0
850	0	0	0	0	0	0	0	0	0	0	0	0	0	0	v	0	v	0	0	+
800	++	0	0	+	v	0	+	v	v	++	v	+	v	v	++	0	+	0	0	0
750	0	0	+	0	0	+	+	0	0	0	0	0	0	0	+	0	0	+	0	0
700	+	v	0	++	0	0	+	0	0	++	v	0	v	0	0	0	0	+	v	+
675	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	+	0	0	0	+
650	0	0	0	++	0	0	+	v	0	0	0	0	0	0	0	v	0	++	0	+
600	0	0	0	0	+	0	+	v	0	0	+	0	0	v	v	0	+	0	0	0
550	0	0	0	++	++	0	0	v	0	0	0	0	0	0	+	0	+	0	v	0
500	++	0	0	0	+	0	0	v	++	0	0	0	0	0	0	0	0	0	0	v
450	0	0	+	++	0	0	0	0	0	0	0	0	0	v	0	0	0	0	0	0
400	++	++	0	0	0	+	+	++	++	0	0	0	0	+	0	v	0	0	+	0
350	0	v	0	0	0	0	0	v	*	0	0	0	0	0	v	0	+	0	0	++
325	0	0	v	0	0	0	0	0	*	0	0	0	0	v	+	0	0	0	++	0
300	0	0	0	0	0	0	0	0	*	0	0	0	0	0	++	0	0	0	0	0
275	0	0	0	0	0	0	0	0	*	0	0	0	0	0	0	0	0	0	0	0
250	0	0	0	0	0	0	0	+	*	0	0	0	0	0	0	0	v	0	0	+
225	0	0	+	0	0	0	+	0	*	0	0	0	0	0	0	0	v	0	v	0
200	0	v	0	0	0	0	+	v	*	0	0	0	v	v	0	0	0	0	v	0
150	0	0	0	0	0	0	0	0	*	0	0	0	0	0	++	0	0	0	0	0
100	+	v	v	0	0	0	+	v	++	++	0	0	0	v	0	0	v	+	0	+

Table 5.0 E 'Turoa'

Polymorphic bands (bp)	Primers from Operon kit C																			
	C-01	C-02	C-03	C-04	C-05	C-06	C-07	C-08	C-09	C-10	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18	C-19	C-20
2000	0	0	0	0	v	0	0	0	0	0	0	v	0	0	0	+	v	0	0	0
1900	++	+	0	0	v	0	0	0	0	0	0	+	0	0	+	0	+	0	v	++
1800	0	0	0	0	+	0	+	0	0	0	0	+	+	0	+	0	0	0	v	0
1700	0	0	0	v	v	v	0	v	0	0	0	+	0	0	++	+	0	+	+	0
1600	v	v	0	0	0	v	+	0	0	0	0	+	+	0	+	0	0	+	0	0
1500	0	0	v	v	0	v	+	0	+	0	0	+	0	0	0	0	0	++	0	0
1400	0	v	v	0	0	0	+	0	+	0	0	+	++	0	0	0	0	0	0	v
1300	0	0	0	+	0	0	++	0	+	0	0	+	0	++	v	0	+	0	0	+
1200	0	v	v	0	0	0	0	v	0	+	0	+	0	0	v	+	0	0	+	0
1150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0
1100	0	0	0	+	0	+	0	0	0	++	v	++	++	v	0	0	0	0	0	v
1000	++	0	0	0	0	++	+	+	0	++	0	++	v	v	v	0	0	++	0	v
950	0	0	0	0	0	v	0	0	0	0	0	+	0	0	+	0	0	0	0	0
900	+	++	++	v	+	0	+	v	0	0	0	+	0	0	0	0	0	v	+	v
850	0	0	0	+	++	0	+	v	0	0	0	0	0	0	v	+	0	v	0	+
800	0	+	+	+	++	0	+	0	0	0	++	+	++	v	0	0	0	+	0	+
750	0	0	0	0	0	v	0	0	0	0	*	0	0	0	0	+	0	v	0	0
700	++	0	0	++	0	v	0	++	0	0	++	v	0	0	0	0	0	0	0	0
675	v	0	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0	0	0	0
650	0	+	+	++	0	0	0	0	0	0	0	0	0	0	+	++	v	0	0	0
600	0	v	+	0	0	0	+	0	0	0	v	v	+	0	0	+	v	0	0	+
550	0	0	++	v	0	+	0	0	0	0	0	0	0	0	0	0	0	v	0	0
500	0	0	+	0	0	+	0	+	0	0	0	0	+	0	0	0	0	+	0	v
450	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	v
400	0	0	0	0	v	0	v	0	0	0	0	0	0	0	0	0	+	0	++	++
350	0	0	0	0	+	v	0	0	0	v	0	0	0	0	0	0	++	0	++	0
325	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	++	0	0	0
300	0	0	++	0	0	0	0	v	v	0	0	0	0	0	0	0	v	v	0	+
275	0	0	0	0	0	0	0	0	0	0	0	0	0	0	v	0	v	v	+	0
250	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	v	0	++
225	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
200	0	v	0	0	v	0	v	0	0	0	0	0	0	0	v	0	0	0	0	0
150	0	0	0	0	v	+	0	0	0	0	0	0	0	0	0	v	0	0	v	0
100	v	0	0	0	0	0	0	0	0	0	0	0	0	0	0	v	0	+	v	0

Table 5.0 F1 'UC157_5'

Polymorphic bands (bp)	Primers from Operon kit C																			
	C-01	C-02	C-03	C-04	C-05	C-06	C-07	C-08	C-09	C-10	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18	C-19	C-20
2000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1900	0	v	0	0	0	0	0	V	0	0	0	0	0	0	0	0	0	+	0	0
1800	++	v	0	v	0	v	0	0	*	0	0	+	0	0	0	0	0	0	0	++
1700	+	0	0	v	0	v	0	0	*	0	0	0	0	0	+	++	++	+	0	0
1600	0	0	v	0	++	0	0	0	*	0	0	+	0	0	++	0	0	0	0	0
1500	0	0	+	+	+	+	0	0	*	0	0	++	0	+	0	++	0	0	0	+
1400	0	0	0	v	+	0	+	0	++	0	0	+	0	0	0	++	+	++	0	0
1300	0	0	+	++	+	0	+	0	0	0	0	0	0	0	++	+	0	v	0	0
1200	v	0	0	++	+	+	+	0	0	0	0	+	+	++	0	v	0	0	++	+
1150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1100	0	0	v	0	0	+	0	0	0	+	+	+	0	0	+	v	0	0	0	v
1000	0	0	0	+	++	+	0	0	0	++	v	++	0	0	v	++	v	++	+	+
950	v	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
900	0	v	++	0	0	0	0	+	0	0	0	+	0	0	v	0	v	0	v	0
850	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
800	+	0	0	++	++	+	v	++	+	0	0	+	v	v	++	+	v	0	+	0
750	0	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0	0	0	0	0
700	0	0	v	0	++	v	0	0	0	v	++	0	v	0	0	+	v	0	0	0
675	0	0	0	v	0	0	0	+	0	0	*	0	0	0	0	0	0	0	0	0
650	0	+	+	0	0	0	0	0	0	0	*	0	0	0	0	0	0	0	0	+
600	0	++	0	+	++	v	0	0	0	0	*	0	v	0	0	+	+	0	0	+
550	++	0	0	0	0	+	0	0	++	0	*	0	0	0	++	0	0	0	0	0
500	0	0	++	++	++	0	0	0	0	0	++	0	0	0	0	0	+	0	+	v
450	0	0	v	0	0	0	0	0	++	0	0	0	0	0	++	0	0	0	0	+
400	0	0	0	++	++	0	0	0	*	v	0	0	0	0	++	0	0	0	0	0
350	0	0	0	0	++	v	v	0	*	0	0	0	0	0	++	0	0	++	0	0
325	+	0	0	0	0	v	0	0	*	0	0	0	0	0	0	v	0	0	0	0
300	0	0	0	0	++	0	0	0	*	++	v	0	0	0	0	0	v	0	0	0
275	0	0	v	+	0	0	0	0	*	0	0	0	0	0	0	0	0	0	0	0
250	0	0	++	0	0	0	0	0	++	0	0	0	0	0	++	v	++	++	0	0
225	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	+	0	0
200	v	v	0	0	++	++	0	0	0	v	0	0	0	0	++	0	++	0	0	0
150	0	0	0	0	v	+	0	0	0	v	0	0	0	0	0	0	0	0	0	0
100	0	0	0	0	v	0	0	0	0	v	0	0	0	0	0	0	++	0	0	0

Table 5.0 F2 'UC157_6'

Polymorphic bands (bp)	Primers from Operon kit C																			
	C-01	C-02	C-03	C-04	C-05	C-06	C-07	C-08	C-09	C-10	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18	C-19	C-20
2000	++	v	0	0	+	v	0	0	0	0	0	v	0	0	0	0	0	0	0	++
1900	++	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	++	0	0	0
1800	+	0	0	0	v	0	0	0	v	0	0	0	0	0	0	0	0	0	0	0
1700	0	v	v	0	0	0	0	0	++	0	0	0	0	+	v	0	0	0	0	v
1600	0	0	0	0	0	0	+	0	*	0	0	0	0	0	+	++	0	+	0	0
1500	0	0	0	v	0	0	0	0	*	0	0	v	0	0	0	v	v	++	++	0
1400	0	0	0	++	+	+	0	0	*	0	0	+	v	0	0	0	0	0	0	0
1300	v	0	0	0	++	0	+	0	++	0	0	0	0	++	++	0	0	0	0	0
1200	0	0	0	v	v	0	0	0	0	0	0	0	0	0	++	0	0	0	0	0
1150	0	0	v	0	v	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1100	0	0	v	0	0	v	v	0	0	0	0	v	+	++	0	0	0	0	0	0
1000	0	+	0	++	0	++	0	0	0	0	0	++	+	++	+	v	0	0	+	0
950	v	0	+	0	0	0	0	v	0	v	0	++	0	0	v	0	0	+	+	0
900	v	0	v	0	++	0	++	+	0	v	0	0	+	0	v	v	0	v	+	v
850	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
800	++	++	0	++	++	++	0	++	0	++	v	++	v	0	0	0	v	0	0	0
750	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
700	v	+	v	++	v	0	+	v	0	++	v	+	0	+	0	0	+	0	0	v
675	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
650	0	++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
600	v	++	0	0	0	+	+	++	++	0	0	+	0	0	0	+	+	0	v	0
550	0	0	0	0	0	0	0	0	*	0	++	0	+	0	v	0	0	0	0	0
500	v	0	v	++	0	0	+	v	*	0	0	0	v	+	+	0	0	++	0	0
450	0	0	0	++	0	0	0	0	*	0	0	0	0	0	v	0	0	0	0	+
400	v	+	0	0	0	0	++	0	*	0	0	0	0	+	++	0	+	0	0	0
350	0	+	0	0	v	0	0	0	*	0	0	0	0	+	0	0	+	0	0	0
325	0	0	0	0	+	0	0	0	*	0	0	0	0	v	0	0	0	+	0	0
300	v	0	v	v	0	0	++	v	++	0	0	0	v	0	0	0	+	0	0	0
275	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
250	0	0	0	0	0	0	0	0	0	v	0	0	0	0	v	0	0	0	0	0
225	0	0	v	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+
200	+	+	0	v	0	v	+	0	0	v	0	v	+	v	0	0	+	+	0	0
150	0	0	0	0	0	0	v	0	0	0	0	0	0	0	0	0	0	0	0	0
100	v	0	0	+	0	0	0	0	0	++	v	0	0	v	+	0	0	v	0	0

Table 5.0 F3 'UC157_11'

Polymorphic bands (bp)	Primers from Operon kit C																			
	C-01	C-02	C-03	C-04	C-05	C-06	C-07	C-08	C-09	C-10	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18	C-19	C-20
2000	0	0	0	v	0	0	0	v	0	v	0	0	0	0	v	+	0	0	0	0
1900	v	0	0	0	0	0	0	0	0	v	0	0	0	0	0	0	0	0	0	0
1800	v	0	0	0	0	0	0	v	0	v	0	0	v	0	0	0	0	0	v	0
1700	+	0	0	v	0	0	0	0	v	0	0	+	+	v	++	0	0	0	v	0
1600	0	0	v	v	0	0	0	0	0	0	0	0	+	0	++	0	0	0	v	0
1500	0	0	v	+	+	0	+	0	0	0	v	0	++	0	0	0	0	++	+	v
1400	0	0	v	0	+	0	v	0	0	0	0	+	0	0	0	0	+	+	0	v
1300	+	0	0	0	0	0	++	v	++	0	0	0	0	v	0	v	0	0	0	0
1200	++	0	0	0	0	0	v	0	*	0	0	+	0	0	v	0	0	0	0	0
1150	0	0	0	+	0	0	0	0	*	v	0	0	0	0	+	++	++	0	v	0
1100	0	0	0	0	v	0	0	0	*	+	0	v	0	0	0	++	+	0	v	0
1000	++	0	0	0	++	0	+	+	*	0	0	++	0	+	0	0	v	0	+	0
950	v	0	0	v	0	0	v	0	*	0	++	0	v	+	0	0	0	v	0	0
900	++	+	+	+	0	0	++	0	*	0	0	+	0	+	0	0	v	+	0	0
850	0	0	0	0	0	++	0	0	++	++	0	0	0	0	0	0	v	++	0	v
800	+	0	++	0	0	++	+	++	0	0	0	++	0	0	v	0	+	++	0	0
750	0	0	0	0	+	+	0	0	0	0	0	0	+	0	0	+	0	0	0	0
700	0	++	+	+	+	0	+	+	0	0	+	0	++	0	0	+	0	0	0	+
675	0	0	v	0	+	0	0	0	0	v	0	0	v	0	++	v	0	0	0	0
650	0	0	v	0	0	0	0	0	0	0	0	0	0	0	+	v	0	v	0	0
600	+	v	v	0	0	0	0	v	0	0	0	0	0	0	0	v	0	+	0	0
550	0	0	0	0	v	v	0	v	++	0	v	0	0	v	v	v	0	+	0	0
500	0	+	+	0	v	v	+	v	*	0	v	0	0	0	v	++	++	0	+	+
450	0	0	0	v	v	0	0	0	*	v	v	0	0	0	+	*	+	+	+	v
400	v	v	0	+	0	0	++	v	*	0	0	0	+	0	0	*	0	0	0	v
350	0	++	0	0	0	0	+	+	*	0	0	0	+	+	0	++	0	0	v	v
325	v	0	v	0	0	+	0	0	*	0	0	0	0	0	0	0	0	+	0	v
300	0	v	v	0	+	+	v	0	++	0	0	0	v	0	0	0	0	0	0	v
275	0	0	0	v	0	0	0	0	0	+	0	0	++	0	0	v	0	0	v	0
250	0	0	0	v	0	0	0	0	0	0	0	0	v	0	0	v	0	0	v	0
225	0	0	v	0	0	0	0	0	0	0	0	0	v	0	0	0	0	0	0	0
200	+	0	0	+	0	0	0	+	0	0	0	v	v	v	0	0	0	0	0	0
150	v	+	0	0	0	v	0	0	v	0	0	0	0	+	0	v	0	v	0	v
100	0	0	0	v	++	0	+	0	0	v	0	0	0	++	0	+	0	v	v	v

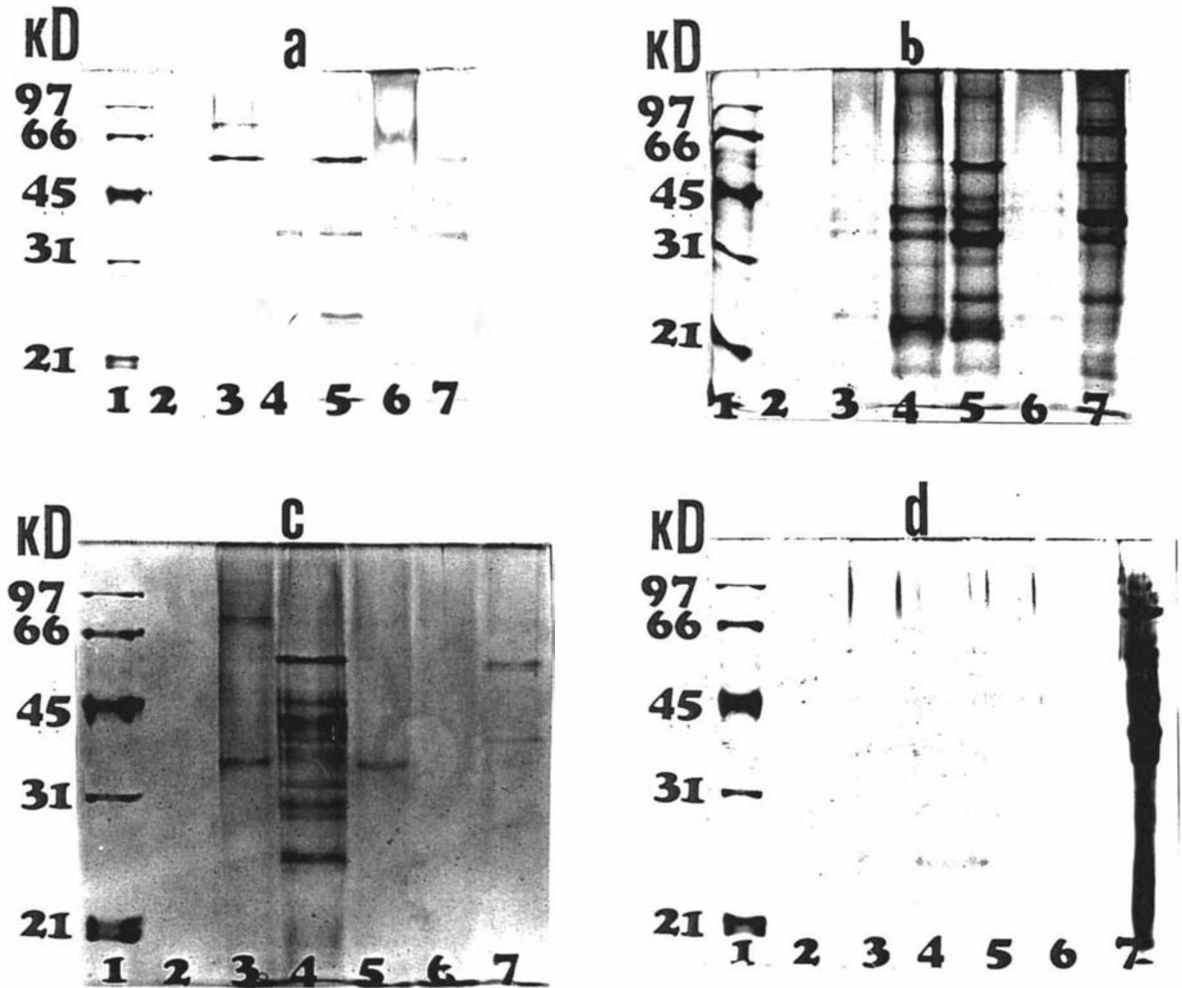

Plate 5.7 Extracellular protein profile of *Asparagus officinalis* L. embryogenic cells grown in suspension culture

Cultivars Aspiring (a), Turoa (b), Karapiro (c), and Pacifica (d). Lane 1 - molecular weight marker, Lane 2 - control, and Lanes 3-7 - protein profiles of embryogenic cells derived from calli grown on 1.0 µM NAA/0.1 µM Kinetin, and 0.3, 1.0, 3.0 and 10 µM 2,4-D.

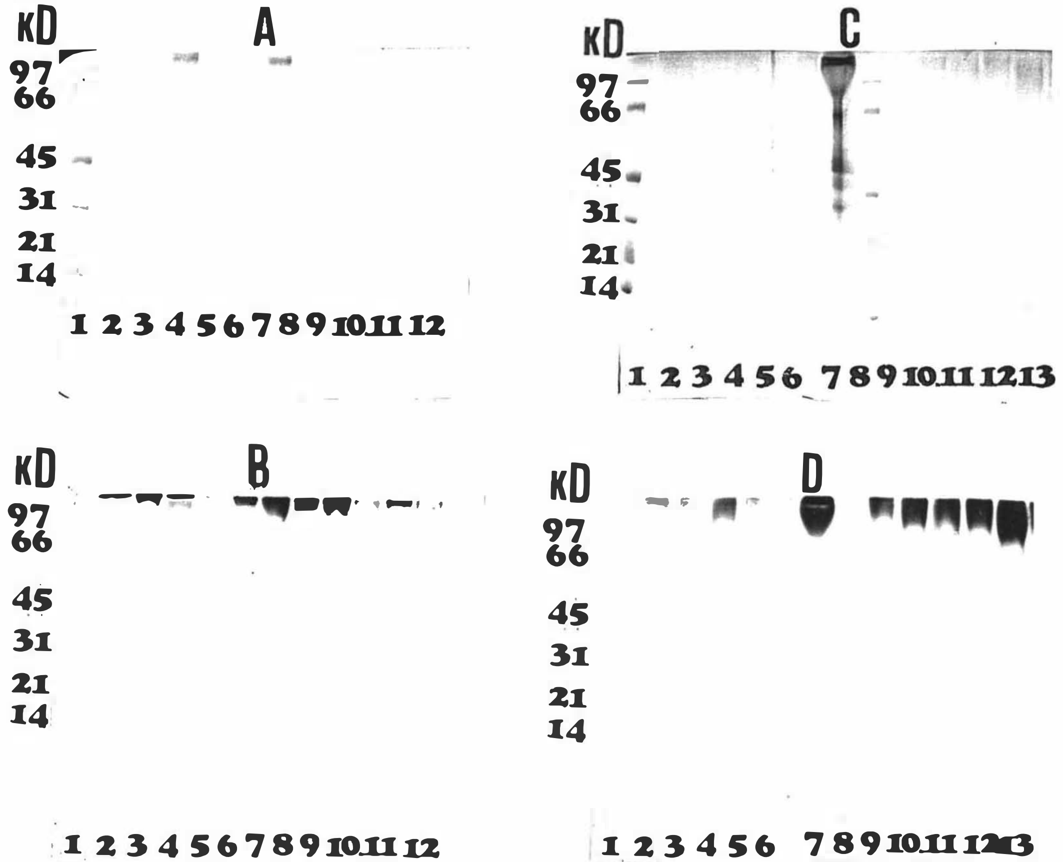

Plate 5.8 Total extracellular protein profiles of asparagus embryogenic suspension cultures

Total extracellular proteins (a) and glycoproteins (b) secreted by embryogenic cells grown in liquid embryo induction medium. Gels in (a) were stained with Comassie Brilliant Blue (CBB). Lane 1 (molecular weight marker), Lane 2-6 - protein profile of cultivar Pacifica embryogenic cells initiated on medium supplemented with 0.3, 1.0, 3.0 and 10 μM 2,4-D and 1.0 μM NAA/0.1 μM Kinetin. Lanes 7-11- protein profile of cultivar Turoa embryogenic cells derived from calli initiated on medium containing 0.3, 1.0, 3.0 and 10 μM 2,4-D and 1.0 μM NAA/0.1 μM Kinetin. Lane 12 - control. The CBB stained gel from (a) was destained and silver stained to detect glycoproteins.

Gel (c) was stained with CBB. Lane 1 molecular weight marker, Lane 2 - control, Lanes 3-7 (cultivar Aspiring) and Lanes 9-13 (cultivar Karapiro) embryogenic cells derived from calli initiated on medium containing .3, 1.0, 3.0 and 10 μM 2,4-D and 1.0 μM NAA/0.1 μM Kinetin. Lane 8 - molecular weight marker. The CBB stained gel from (c) was destained and the silver stained (d) to detect glycoproteins.