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Production of doubled haploid plants in Brassica oleracea

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Project Number: VG00004

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The research contained in this report was funded by Horticulture Australia Ltd with the financial support of the Vegetable Industry.

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Know-how for Horticulture™

Investigating doubled haploid plant development in

Australian varieties of cauliflower

(*Brassica oleracea* var. *botrytis*).

By

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The purpose of the report

At present, open pollinated varieties of cauliflower have been largely substituted by F1 hybrid varieties in Australia and around the world. Conventional techniques of developing hybrid seeds are highly time consuming and have limited the efficiency of new hybrid variety and cultivar development. Thus, the current cauliflower hybrid seed industry demands an efficient, rapid, and reliable technique for developing hybrid varieties. Doubled Haploid (DH) technology is an alternative approach that may accelerate hybrid seed production. In cauliflower, the current techniques of developing DH are still in its infancy, despite considerable attempts. The present study was initiated with the objective of establishing an efficient DH technique for Australian varieties of cauliflower. This report summarises the outcomes of this investigation particularly on the anther culture technique.

CONTENTS

MEDIA SUMMARY

Most of the cauliflower (*Brassica oleracea*) varieties grown around the world are F1 hybrids. Hybrid varieties offer consistency of vigour, yield and quality, and are produced via the crossing of two highly inbred parental lines. At present, a major concern among cauliflower breeders is the lack of a reliable and rapid method for producing inbred lines, as conventional inbreeding techniques are labour intensive and time consuming. In this project, the feasibility of using anther culture as an alternative and faster method of producing inbred (pure) lines was studied.

Anther culture involves the removal of anthers, which bear immature pollen, from donor cauliflower plants, and subsequently culturing them on nutrient media under sterile conditions. If the procedure is successful, the immature pollen, which contain only half the number of chromosomes (haploid) compared to a normal plant (diploid), will undergo cell division and produce haploid plants. The resultant plants may be converted into diploid plants through the use of chemicals, or this conversion may occur spontaneously during culturing. Diploid plants produced in this manner (doubled haploids) are pure breeding, and are comparable to inbred lines produced by many generations of self pollination.

The major aims of this project were to adapt, and optimise procedures developed for the anther culture of other *Brassica* crops for Australian cauliflower varieties. Three F1 hybrid varieties (Cashmere, Candid Charm and Snow Mountain) were obtained from Henderson Seed Group Pty. Ltd. and were subsequently used in all experiments. Optimisation of anther culture conditions involved experiments on discovering the optimum concentrations of certain nutrients and regulatory factors, for example, sucrose and silver nitrate. It also involved the optimisation of culture temperature and other environmental factors.

Results from these studies indicated that all three Australian varieties used were amenable to anther culture to varying degrees; the best results were obtained with the variety "Snow Mountain". Under optimised culture conditions, we were able to obtain 93 plants for every 100 anthers cultured. While this frequency of doubled haploid plant production is acceptable for practical breeding purposes, further research is necessary, especially in the methods used for confirming the quality of the plants produced.

TECHNICAL SUMMARY

Most of the cauliflower (*Brassica oleracea*) varieties grown around the world are F1 hybrids. Hybrid varieties offer consistency of vigour, yield and quality, and are produced via the crossing of two highly inbred parental lines. At present, a major concern among cauliflower breeders is the lack of a reliable and rapid method for producing inbred lines, as conventional inbreeding techniques are labour intensive and time consuming. In this project, the feasibility of using anther culture as an alternative and faster method of producing inbred (pure) lines was studied.

The major aims of this project were to adapt, and optimise procedures developed for the anther culture of other *Brassica* crops for Australian cauliflower varieties. Three F1 hybrid varieties (Cashmere, Candid Charm and Snow Mountain) were obtained from Henderson Seed Group Pty. Ltd. and were subsequently used in all experiments. Optimisation of anther culture conditions involved experiments on discovering the optimum bud size for anther extraction, and concentrations of certain nutrients and regulatory factors, for example, sucrose and silver nitrate. It also involved the optimisation of anther pre-treatments, such as heat/cold shock durations. Further, experiments were conducted to compare the efficacy of different methods of ploidy determination, such as Fluorescent Flow Cytometry (FFC) and PCR-based molecular markers.

Existing protocols developed for *Brassica oleracea* var. *italica* (Broccoli) by Keller and Armstrong (1983) and for *Brassica napus* by Lichter (1981) were used in the initial experiments. Of these two, the protocol by Keller and Armstrong (1983) was found to be more suitable. Subsequent experiments concentrated on optimising this protocol in terms of sucrose and silver nitrate concentration. Bud size experiments indicated that the largest proportion of uninucleate microspores, which are most amenable to anther culture were found in buds 2-4 mm in length. The sucrose and silver nitrate concentrations which produced the largest number of embryo-like structures (ELS) per anther were 6-10% and 15-30 ppm respectively. Heat shock of anthers before culturing was found to be highly effective in increasing the frequency of ELS formation. Anthers exposed to $35^{o}C$ for 24-48 hours were significantly more responsive to anther culture (by up to 60-70%) compared to the control (no heat shock). The FFC technique for ploidy determination was effective in discriminating between haploids, diploids, tetraploids and aneuploids. However, it could not discern between diploids arising from spontaneous endoreduplication from those arising from contaminating diploid tissues. The PCR-based ploidy determination experiment was unsuccessful, due to the lack of polymorphisms between the inbred parental lines for the microsatellite loci assayed.

Results from these studies indicated that all three Australian varieties used were amenable to anther culture to varying degrees; the best results were obtained with the variety "Snow Mountain". Under optimised culture conditions, we were able to obtain 93 plants for every 100 anthers cultured. While this frequency of doubled haploid plant production is acceptable for practical breeding purposes, further research is necessary, especially in the methods used for confirming the ploidy of the plants produced. Further, other techniques for producing doubled haploids, such as isolated microspore culture and gynogenesis should be investigated for cauliflower. Although such work was initiated as part of this project, insufficient information was obtained on their efficacy.

INTRODUCTION

Cauliflower (*Brassica oleracea* var. *botrytis*) and other common vegetable crops including cabbage, broccoli, kohlrabi and Brussels sprouts are collectively recognised as Cole crops (Phillips, 1995; Rae *et al*., 1999; Rudolf *et al*., 1999). These crops are primarily utilised for human consumption. Around the world, cultivated *Brassica* consumed as vegetables (especially *B. oleracea*) represent the second largest harvested production among vegetable crops. Cauliflowers are highly appreciated and much consumed as a vegetable. In Australia, cauliflowers are commercially grown in all states but are best suited to the cooler climates of southern states (Phillips, 1995). Over the past years cauliflower production in Australia increased from 50,000 tonnes in 1975 to 120,000 tonnes in 2002, of which 33,070 tonnes were exported (FAO, 2003). This growth in the production was due partly to improved agronomic practices and the use of high yielding, disease resistant new F1 hybrid varieties. Open-pollinated varieties of cauliflower have been largely replaced by F1 hybrids throughout Australia and around the world (Phillips, 1995).

Hybrid crop varieties offer many incentives to the grower (e.g. increased vigor, yield and uniformity) and to the breeder (intellectual property protection, guaranteed return seed sales) (Ripley and Beversdorf, 2003). Thus, the production of cauliflower hybrid seeds has become an economically viable industry. Though most of these companies are of European origin, several Australian seed companies also produce cauliflower hybrid seeds for local as well as world markets (Phillips, 1995). At present, a major concern among cauliflower breeders is the lack of a reliable, rapid method for generating parent lines for hybrid combinations, which has hampered the efficient development of new hybrid varieties and cultivars.

Conventionally, F1 hybrid seeds are produced by a hybridisation system, based on self-incompatibility or male sterility of the parent lines (Bhalla and Weerd, 1999). Self-incompatible parent lines (pure lines) for hybrid seed production are developed through a process of selfing *via* bud pollination, which is tedious, and costly. It requires approximately five to six years to establish sufficiently stable homozygous parent lines that may be used in hybrid seed production (Farnham, 1998; Saji and Sujatha, 1998; Lesniewska *et al*., 2001). Additionally, traditional breeding methods are unable to produce completely homozygous plants even with infinite numbers of repeated self-pollinations (Chekurov and Razmakhnin, 1999).

An alternative approach is to integrate doubled haploid (DH) technology into breeding programmes. This technique provides plant breeders with pure lines (with 100% homozygosity), in a single generation as opposed to traditional plant breeding (Tuvesson *et al*., 2000), facilitating the development of hybrid seeds and new cultivars in a comparatively shorter time (Barro and Martin, 1999; Barro *et al*., 2001; Achar, 2002). Introduction of this technique into several plant breeding programmes had reduced the time and population sizes required for the production of pure lines compared to the conventional methods of selfing or backcrossing (Foroughi-Wehr and Wenzel, 1990; Guzy-Wrobelska and Szarejko, 2003). It has been revealed that this technique could even be employed as effectively as the pedigree method for developing cultivars resistant to various plant diseases, with savings in time and labour (Martinez *et al*., 1996). Furthermore, it was reported that DH plants permit the acceleration of production and marketing of new varieties by up to 50% (Pechan and Smykal, 2001).

Cauliflower had received less attention compared to other species and varieties of *Brassica* with regard to the DH technique. For the efficient application of this technique, sufficient numbers of DH regenerants should be achieved from a broad spectrum of genotypes (Rudolf *et al*., 1999). One of the main shortcomings in cauliflower is the existence of high genotypic specificity

(Fuller and Turton, 1990; Yang *et al*., 1992; Stipic and Campion, 1997). Additionally, the majority of genotypes are somewhat recalcitrant (Phippen and Ockendon, 1990; Stipic and Campion, 1997) and post-embryonic difficulties, such as poor regeneration (development of haploid embryos to plants) has been reported. Thus, the current techniques are considered to be inefficient, inconsistent or unreliable and have thus far limited the exploitation of this technique in commercial cauliflower breeding programs.

The significance of doubled haploids (DH) in the discipline of plant breeding and genetics is widely acknowledged and well established (Maheshwari *et al*., 1982; Chen and Beversdorf, 1992; O' Donoughue and Bennett, 1994; Chaudhary *et al.,* 2003). In plant breeding, DH facilitates the rapid production of pure breeding lines that can be evaluated for yield, quality, disease-resistance and for many other agronomic attributes (Ferrie *et al*., 1999; Immonen and Anttila, 1999; Lionneton *et al*., 2001). DH lines are the perfect starting material in generating new F1 cultivars (Zamani *et al*., 2000). In DH lines, homozygosity is higher than in inbred lines and thus, hybrids between two DH lines are often superior to those between inbred lines in their yield, growth, vigor and uniformity (Pauk *et al*., 1995; Baenziger, 1996).

One of the recent benefits of this technique is its applications to the discipline of plant genetics. In the field of genetics, DH have been used in the construction of molecular maps, to develop molecular markers, to examine the number of genes, type of gene action, heritability of a trait and to increase the genetic variation available for a particular trait (Uzunova and Ecke, 1999; Betty *et al*., 2000; Chalyk and Chebotar, 2000; Chen *et al*., 2001). Thus, exploitation of DH in cauliflower breeding and genetic programmes will contribute to; the rapid development of homozygous lines, the reduction in the breeding time of new cultivars and in enhancing our knowledge of agronomically significant genetic traits.

Doubled haploids are of great value in other disciplines including evolutionary and mutation studies, cytogenetics, plant pathology, plant physiology, biotechnological manipulations and germplasm studies (Bansal *et al*., 1999; Rae *et al*., 1999; Smykal and Pechan, 2000; Barro *et al.,* 2001; Pechan and Smykal, 2001). Although the potential benefit of doubled haploids seems to be considerable, this technique is yet to be applied to many of these areas except in plant breeding and genetic studies where good progress have been made (Maheshwari *et al*., 1982). Hence when developed, cauliflower DH may not only assist in plant breeding and genetics, the main emphasis of this study, but may also initiate many new avenues of research, such as molecular mapping, evolutionary and mutation studies which will undoubtedly support the long term viability of this agricultural enterprise. Thus, the present study was initiated with the following objectives.

1. To study the amenability of Australian varieties of cauliflower to androgenesis with existing protocols *via* anther culture.
2. To optimise a selected anther culture protocol with reference to several cultural factors.
3. To evaluate androgenic regenerants for their ploidy levels.

MATERIALS AND METHODS

1. Investigating the amenability of Australian varieties of cauliflower to androgenesis *via* anther culture.

1.1.Protocols screened

Protocol 1 - by Keller, W. A and Armstrong, K. C. (1983). Production of haploids *via* anther culture in *Brassica oleracea* var. *italica* L. and
Protocol 2 - by Lichter, R. (1981). Anther culture of *Brassica napus* L. in liquid culture medium (these protocols are detailed in Table 2.1).

1.2. Plant material

Three cauliflower genotypes, obtained from Henderson Seeds Group Pty Ltd. (Templestowe, Australia) were used as anther donors. They were the Cashmere (9791571), Snow Mountain (330p) and Candid Charm (971572). All three genotypes were F1 hybrids.

1.3. Seed germination and raising donor plants

Seeds were sown into three separate plastic trays (each genotype into one tray) filled with potting mixture (ready-made potting mix- Hortico^{TM}) and finely chopped Osmocote$^®$ granules (1g). Trays were kept inside a misting chamber (100% RH) for about three weeks. Seedlings were transplanted to 20 cm plastic pots (containing same potting mix) and raised in a glasshouse (Section 1.4.2). Initially, one pot contained two plants and after one month they were thinned to one plant per pot. Due to limitations in glasshouse space, and management difficulties, only six plants per genotype were raised, giving a total of 18 donor plants. This was not a problem experimentally, as each plant produced several hundred flower buds. Pots were arranged in a completely randomised manner. Seeds were sown sequentially every three months to ensure a continual supply of flower buds.

1.4. Growing conditions

1.4.1. Soil and fertiliser regimes

The potting mixture utilised was a ready-made potting mix (Hortico^{TM}). Plants were fertilised with Aquasol ^{TM} (N.P.K. 23: 4: 18) in conjunction with Osmocote$^®$ (N.P.K 5: 5.2: 12.5). Aquasol was applied once a week (120 ml per pot per application), as directed by the manufacturer. Approximately 1g of Osmocote$^®$ per pot were applied at three-weekly intervals. The surface of the soil was worked in every three weeks to improve aeration. Plants were watered three days per week.

1.4.2. Glasshouse conditions

Plants were grown to flowering under glasshouse conditions. Temperature was controlled within the range of 20-23^{0}C and photoperiod was maintained at 12 h with high-pressure sodium bulbs and fluorescent tubes (220 μmol m^{-2} s^{-1}).

1.4.3. Pest and disease control

This was accomplished by applying Pyrethrum (Multi Crop Aust. Pty. Ltd.) and Benlate® when necessary. To improve inflorescence development and to reduce the spread of diseases large sections of the curds were removed before bolting.

1.5. Bud harvesting

Approximately three months after seed germination, plants were ready for bud collection. For each genotype, floral buds (FB) were gathered from six donor plants, and then were bulked to generate a representative sample. Each plant was only sampled twice for buds. FB, sizes ranging between 2-3 mm were harvested when the first three FB in the inflorescences were fully opened. Preliminary investigations indicated that this size class contains predominantly uninucleate microspores, which are reported as highly amenable to androgenesis. They were gathered prior to the experiment, on to a cold beaker usually in the morning (8-10 am).

1.6. Anther culture

1.6.1. Media preparation and composition

Media compositions for both protocols are detailed in Table 1.1. Unless specifically stated all ingredients were purchased from Sigma-USA. Potato extract for Protocol 2 was made by boiling 100 g of fresh potato tubers in 250 ml of water, mashing the boiled tubers and filtering the slurry through muslin cloth. Media was prepared using MilliQ water (Millipore, Millipore Inc., France) and the pH was adjusted with 1M NaOH to 5.8. For solid media, 0.8% agar (Bacteriological-Oxoid) was added after the pH adjustment. Sterilisation was by autoclaving at 121^0C for 20 minutes. Plant growth regulators were filter-sterilised (0.45 µm, Gelman Science, Acrodisc® 32) and was added to the media after autoclaving. Prepared media were stored at 4^0C for about one-month prior to use.

Table 1.1. The base, and compositions of the media.

PROTOCOL	BASIC SALTS AND VITAMINS	OTHER INGREDIENTS	
Protocol 1	Gamborg B5 (1968)	Glutamine	5.4 mM
		2,4-D	0.4 µM
		NAA	0.5 µM
		Sucrose	10%,
		Agar	0.8%
		pH	5.8 ± 0.1

PROTOCOL	BASIC SALTS AND VITAMINS	OTHER INGREDIENTS
Protocol 2	Nitsch and Nitsch (1969)	Glutamine 5.4 mM Glutathione 97 µM L-Serine 0.95 mM NAA 2.6 µM BAP 0.04 µM Potato extract ~ 2.5 g/L, Sucrose 8% pH 5.8 ± 0.1

1.6.2. Sterilisation and isolation of anthers

Surface sterilisation of buds was accomplished by immersing flower buds in a freshly made solution of sodium hypochlorite (10% w/v) for 15 minutes followed by three sequential washes in sterile water. Using fine forceps and needle flower buds were dissected and anthers were carefully removed without their filaments. This was achieved with the aid of a dissecting microscope and extreme caution was taken not to damage anthers. Surface sterilisation, bud dissection, and anther plating were performed in a sterile environment provided by a laminar flow cabinet (HWS series-CLYDE-APAC).
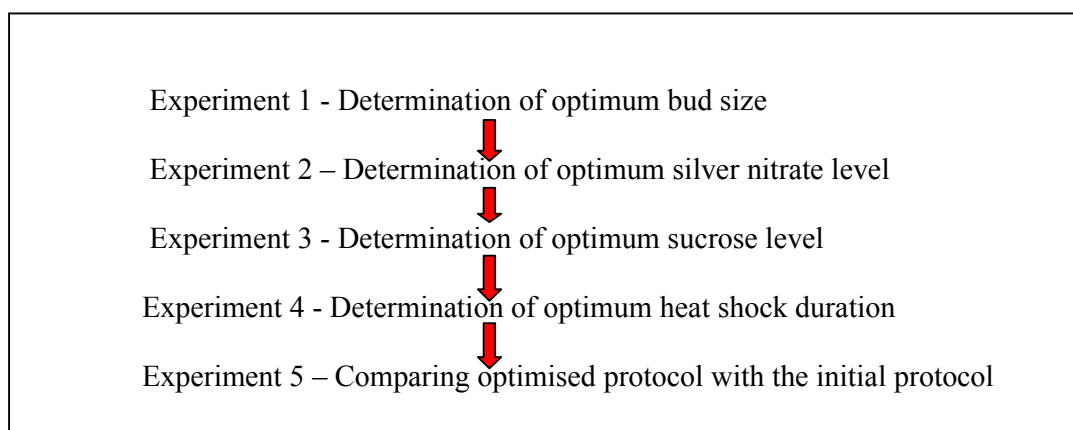
1.6.3. Anther plating and incubation

Six anthers from each bud were plated onto each petri dish containing 15 ml of culture medium with the aid of a small paint brush (No 2). Anthers were distributed evenly on the culture plate. In total, 306 anthers were cultured for each protocol. Plated AC plates were sealed with two strips of Parafilm (PARAFILM "M" ®-American National Can TM) and were covered with aluminum foil. Immediately after plating, the culture plates were subjected to the incubation conditions specified by each protocol (Table 1.2). Both protocols specified the high temperature initial induction period, followed by the post incubation period at 25^0C, which was reported as essential for *Brassica* (Table 2.2). During the initial induction period, culture plates were kept in the dark while during the post culture period they were exposed to 16 h photoperiod provided by fluorescent tubes. After five weeks of incubation each culture plate was scored for the numbers of induced anthers in terms of the embryo-like structures (ELS) and callus formation.

Table1.2. Incubation conditions for the each protocol.

PROTOCOL	INITIAL INCUBATION	POST INCUBATION
Protocol 1	35^0C, 24 h, dark	25^0C, 34 days, 16 h photoperiod
Protocol 2	30^0C, Three weeks, dark	25^0C, Two weeks, 16 h photoperiod

10

2.   Sequential optimisation of an anther culture protocol for cauliflower for four cultural factors

For this experiment, unless specifically stated, plant material (1.2), seed germination and raising donor plants (section 1.3), growing conditions (section 1.4) and bud harvesting (section 1.5) were as described for the section 1. Anther culture medium (Table 1.1) and incubation conditions (Table 1.2) were as for Protocol 1 unless specifically stated. Surface sterilisation, isolation of anthers, anther plating and incubation were as described under the section 1.6.2 and 1.6.3. When optimum condition (level) for one cultural factor is determined, that level was employed for subsequent experiments as indicated by the experimental flow chart for the series of experiments (Figure 2.1).

Experiment 1 - Determination of optimum bud size

Experiment 2 – Determination of optimum silver nitrate level

Experiment 3 - Determination of optimum sucrose level

Experiment 4 - Determination of optimum heat shock duration

Experiment 5 – Comparing optimised protocol with the initial protocol

Figure 2.1. Flow chart showing the sequential optimisation of four different cultural factors.

2.1. Determination of optimum bud size

Initially, flower buds were separated into three size classes (based on their measured lengths) as in Table 2.1. Class-1 consisted of bud sizes ranging from 0.5 to 1.5 mm and Class-3, consisted of flower buds ranging from 4.5 to 8.5 mm (Figure 2.2). Two experiments were designed to investigate the effects of bud size on anther/microspore developmental stage and subsequent androgenesis.

Table 2.1. Different bud size classes and their measured bud lengths.

SIZE CLASSES	MEASURED BUD LENGTHS
Class-1	Less than 2 mm
Class-2	2-4 mm
Class-3	Greater than 4 mm
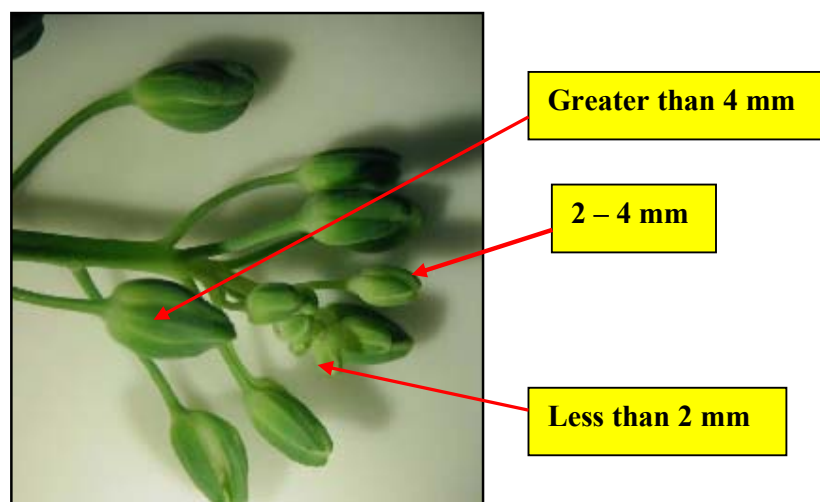
Figure 2.2. Cauliflower inflorescence showing the distribution of different bud size classes.

2.1.1. Experiment one–culture of anthers belonging to various bud size classes

From each genotype, ten flower buds belonging to each bud size class (Figure 2.2) were dissected and their anthers (six anthers) were plated onto petri dishes containing the culture medium described for Protocol 1.

2.1.2. Experiment two–cytological observation of various microspore developmental stages in various bud size classes

For each genotype, ten flower buds from the each bud size class were treated with a fixative (ethanol 6: glacial acetic acid 3: and chloroform 1) for three days. Thereafter each bud was placed on a glass slide and anthers were dissected out. A drop of freshly made 2% acetocarmine stain was added to isolated anthers. Acetocarmine squashes (of the individual anthers) were examined under a light compound microscope (Olympus) with the 100x magnification. Three different fields were chosen randomly for each bud, and various microspore developmental stages were recorded for a hundred microspores within these fields. Developmental stages recorded included, early stages of cell divisions (ESC), tetrads (TT), uninucleate (UNI), binucleated (BINU) and other developmental stages (OTH), which included all cells that were difficult to identify (unclear and unidentified) as belonging to either of the above stages. Guidelines established for *B. napus* (Coventry *et al*., 1988) were employed in identify uninucleate and binucleated microspores (Table 2.2).

Table 2.2. Characteristics of various microspore developmental stages.

Microspore developmental stages	Identifying characteristics
Early uninucleate Mid uninucleate Late uninucleate	Nucleus central, exine pale and thin. Nucleus central, exine thick, well-developed lobes. Nucleus peripheral, exine yellow, spore tri-lobed.
Binucleated	Generative nucleus dense, vegetative nucleus large and diffuse.

2.2. Experiments with silver nitrate, sucrose and heat shock

2.2.1. Determination of the effect of silver nitrate

For this experiment, flower bud sizes ranging from 2-6 mm was utilised. Results from the previous experiment (section, 2.1) indicated that both, Class-2 and 3 flower buds were suitable for the three tested genotypes. Anthers were dissected and were plated onto each petri plate containing the medium described for Protocol 1 except for the different silver nitrate concentrations used, which were 0, 7, 15 and 30 mg/L. Silver nitrate was initially dissolved in three drops of ethanol (70%) and was added to the culture medium prior to sterilisation and pH adjustment. Sterilised culture medium was mixed thoroughly (shaking) before pouring into petri plates to minimise the possible uneven distribution of silver nitrate.

2.2.2. Determination of optimum sucrose concentration

The procedure and conditions for anther culture were as for the above experiment (section, 2.2.1) except, that the culture medium was supplemented with 20 mg/L silver nitrate. Results from the previous experiment (section, 2.2.1) indicated that the addition of silver nitrate had a positive effect and that the optimum level was around 20 mg/L. The different sucrose concentrations tested were 2, 6, 10 and 15%, where as 10% was the concentration described for the Protocol 1.

2.2.3. Determination of optimum heat shock duration

Flower bud size, surface sterilisation, anther plating and incubation were as described for the previous experiment (section, 2.2.2). Culture medium was supplemented with 20 mg/L silver nitrate and 12% sucrose (results from the previous experiment (2.2.2) indicated that, a sucrose level between 10 and 15% was better for the tested genotypes). In this experiment, three different heat shock conditions varying in their durations were tested. They were, 35^0C for 24 h, 35^0C for 48h and 35^0C for 72 h. Anther culture plates kept continuously at 25^0C for five weeks were used as controls for this experiment.

Five weeks after the culture initiation, the numbers of induced anthers were recorded and their corresponding percentages were calculated for the each treatment. Analysis of variance (ANOVA) was performed on untransformed data using the statistical software package Minitab version 12.3 (Minitab inc., USA). In the analysis culture dishes lost due to contamination (fungal, bacterial and mite) were represented by the * symbol.

2.4. The effectiveness of the optimised protocol relative to the initial protocol (Protocol 1)

The culture media and conditions for Protocol 1 and the optimised protocol are listed in Table 2.3. This experiment was laid out in a completely randomised manner with seventeen replicates for each genotype. Each replicate was represented by a petri dish containing six plated anthers from a single bud. In all, 306 anthers were cultured for each protocol (102 from each genotype). Entire replication was completed over a five-day period. After five weeks of incubation, the number of induced anthers for each replicate was recorded and their percentages were calculated. As the residuals from the model were normally and independently distributed analysis of variance (ANOVA) for the treatments (Protocols) were performed on untransformed data using the statistical software package Minitab version 12.3 (Minitab inc., USA). Data was initially analysed for the day effect and was found to be insignificant.

Table 2.3. Conditions varied with the initial (Protocol 1) and the optimised protocol.

PROTOCOL	FACTOR	LEVEL
Protocol 1	Bud size Culture medium Heat shock	2-3 mm Sucrose 10%, Silver nitrate 0 mg/L At 35^0C for 24 h
Optimised protocol	Bud size Culture medium Heat shock	2-6 mm Sucrose 12%, Silver nitrate 20 mg/L At 35^0C for 48 h

3. Regeneration and maturation of embryo like structures (ELS) and callus

Media selected for regeneration and maturation was B5 (Gamborg *et al*., 1968) and MS (Murashige and Skoog, 1962).

3.1. ELS and callus induction

Anthers were induced according to the conditions and procedures described for the optimised protocol. Five weeks after culture initiation, from each anther culture plate, even numbers of induced anthers were removed and were plated equally onto MS (Murashige and Skoog, 1962) and B5 (Gamborg *et al*., 1968). For example, if an anther culture dish was with five induced anthers, four of them were removed and plated onto B5 (Gamborg *et al*., 1968) and MS (Murashige and Skoog, 1962) media, distributing equal numbers per each dish. A total of six induced anthers were plated onto single petri dish. Culture plates were sealed with two strips of Parafilm and were incubated at 24 ± 1^0C and 16 h photoperiod in an illuminated (fluorescent tubes) growth chamber (Thermoline L+M, RI 250SG-370-D). After five weeks of incubation, the following parameters were recorded for each culture plate. They were;

- Number of proliferating calli and the average size of the calli.
- Number of matured embryos and calli with observable root and shoot development.

After three subculturing steps (two with callus and one with plantlets), regenerated plantlets were acclimatised in a glasshouse. The whole regeneration and maturation process consumed between two to three months.

4.   Ploidy level determination of anther derived regenerants for the identification of haploids, diploids (Doubled haploids) and polyploids

Plant materials utilised were the three F1 genotypes (described in section 1.2) and their anther-derived regenerants, developed *via* the protocol established in this study.

4.1. Morphology

Morphological features were recorded for a sample of 30 regenerants from the day of transferring to soil until their maturity in the glasshouse. Observations were made once a week. Characters examined were, leaf morphology, plant habit (height and branching) flower colour, size and fertility. Fertility was assessed by the presence of pollen and the development of fertile pods with selfed seeds.

4.2. DNA flow cytometry

A small sample of twenty, one-month-old, soil-transferred regenerants (4-6 leaf stage) were selected for this study. Selection was based on the leaf morphology (size and shape) and the sample consisted of regenerants with different as well as similar leaf morphologies to their donor plants. The third and fourth leaves from the apex of the plant were detached and was utilised for this experiment. After detachment from the plants, leaf tissues were stored in a freezer (- 20^0C) for three days before the analysis. They were sealed inside a plastic bag with moist (with sterile water) cotton wool. Sample preparation and staining were done as described by Croser *et al*. (2000). For nuclear release, leaf tissue (0.5 g) was chopped in nuclei extraction solution (Partec 06-5-4004) with a sharp razor blade for 30 to 60 seconds and left for 3 minutes. Filtered samples (50 µm filter) were stained with DAPI (DAPI, Partec High resolution DNA staining kit for plants) staining solution and the relative fluorescence of total DNA of single nuclei was measured using a flow cytometer (PA11-Partech, Germany). The instrument was calibrated using three known diploid samples of cauliflower as an internal references and diploid peak intensity was set to value of fifty. Ploidy levels of the regenerants were determined by comparing their histograms with the histograms of internal references.

4.3. Molecular-marker based identification

Due to difficulties encountered in obtaining seeds of the inbred parents for Snow Mountain, Cashmere and Candid Charm, this experiment was split into two phases, phase A and B. The phase A, which was setup to demonstrate the Mendelian inheritance of the markers, consisted of two parental genotypes (section 4.3.1.1) and an F1 hybrid. The phase B consisted of three F1 hybrids used previously in this study (section 4.3.1.2) and their anther-derived regenerants.

4.3.1. Plant material

4.3.1.1. Phase A

The cauliflower genotypes used were, W-01=1042, YI-103=6H158A, and HS 5140-3210/L2. Seeds were obtained from Henderson Seeds Group Pty Ltd. (Templestowe, Australia). W and YI are two parents and HS is the hybrid of those two. After surface sterilisation (by immersing seeds in a freshly made solution of sodium hypochlorite (2%) for 10 minutes followed by three sequential washes in sterile water), seeds of these genotypes were grown on petri dishes

containing moistened filter paper. When they were one week old (seedling stage) leaf tissue was used for DNA extraction.

4.3.1.2. Phase B

Three F1 hybrid cultivars "Snow Mountain", "Cashmere", "Candid Charm" and their anther-derived regenerants were used for this study. The seeds of F1 plants were grown as described for the phase A and leaf tissue (at seedling stage) was used for DNA extraction. Regenerant plants from Snow Mountain, Cashmere and Candid Charm were developed, *via* the anther culture protocol established in this study. Leaf tissue for DNA extraction was gathered from one-month old regenerants.

4.3.2. DNA extraction

Genomic DNA was isolated from leaf tissues of cauliflower using a modified CTAB technique (Taylor *et al*., 1995). For each genotype the leaves of five seedlings were bulked together for the DNA extraction (about 0.1 g of tissue). The DNA concentration was adjusted to a final concentration of 10 ng/µL for each sample, by diluting with sterile MilliQ water and were stored at 4^0C. The quality and quantity of extracted DNA was estimated by gel electrophoresis.

4.3.3. PCR amplification and gel electrophoresis

PCR was performed in a Thermo Hybaid PCR Express Thermal Cycler according to the cycling conditions described by Kresovich *et al*. (1995). Seven SSR primer pairs designed by Kresovich *et al*. (1995) were tested for polymorphisms between the two parents and their anther derived regenerants (Table 4.1). All primers were obtained from GeneWorks Pty Ltd. PCRs were performed in a total volume of 25 µl, containing 30 ng of template DNA, 0.25 mM of each deoxynucleotide, 1 unit of *Taq*–DNA polymerase (Invitrogen-Life Technologies, U.S.A), 1x PCR buffer, 1 µM of each primer and 3.0 µg of $MgCl_2$.

PCR Profile - Forty cycles of, 1 min at 94^0C (denature), 30 S at 63^0C annealing temperature followed by a final extension step of 45 S at 72^0C.

Amplified products were mixed with a 5µl of loading dye (0.25% Bromophenol Blue, 0.25% Xylene Cyanol, 40% sucrose (w/v)) and resolved by gel electrophoresis in precast 10% acrylamide gels in Tris-Borate buffer (Bio-Rad Cat. No. 161-1128), for 90 minutes at 100V. The gels were stained with ethidium bromide and visualised with UV light using a transilluminetor.

Table 4.1. Sequences of *Brassica* SSR Primer pairs.

Primer name	Sequence (5′ ⟶ 3′)	Size
B.n.6A2L	CTT TGY GTG GAC TTT TAG AAC TTT A	25
B.n.6A2R	CGC AGC TTT TGG CCC ACC TG	20
B.n.6A3L	GCT ACC CAC TCA TGT CCT CTG	21
B.n.6A3R	CCA AGC TTA TCG AAT CTC AGG TA	23
B.n.9AL	GAG CCA TCC CTA GCA AAC AAG	21
Primer name	Sequence (5′ ⟶ 3′)	Size

B.n.9AR	CGT GGA AGC AAG TGA GAT GAT	21
B.n.16AL	CGA CCG TGG AAG CAA GTG AG	20
B.n.16AR	CCA TGA TTA CGC CAA GCT ATT TA	23
B.n.18A1L	TCA ATC CCA CCA CCA ACC AGA CAA A	22
B.n.18A1R	TAA GAC AGG TAA GGT TTG CCC C	22
B.n.20AL	GAC AAT CAA TCC CAC CAA CCA G	22
B.n.20AR	TAA AAG AAG AGT GCC AAT CCC AT	23
B.n.25AL	CAC GTG GTA TGT TGG TAT TGG G	22
B.n.25AR	TGA TTC TCC TCC GAC GCA TGC	21

* L and R refer to forward and reverse primers.

RESULTS

1.   Investigating the amenability of Australian varieties of cauliflower to androgenesis *via* anther culture.

Screened protocols were effective in inducing ELS or callus (androgenic responses) in the tested cauliflower genotypes. However, there was a significant difference in the efficiency of the two protocols, in terms of the percentage of ELS/callus induction, where Protocol 1 was found to be superior to Protocol 2. Additionally, results indicated a significant interaction between the protocols and the genotypes. "Snow Mountain" and "Cashmere" gave significantly better androgenic responses to Protocol 1 than to Protocol 2 (Figure 1.1 and Figure 1.2) whereas, the anthers of "Candid Charm" responded equally to the two protocols (Figure 1.2). Although the androgenic response of "Cashmere" to Protocol 2 was marginally higher than to Protocol 1, androgenic ability of "Cashmere" was comparatively lower than either to "Snow Mountain" or "Candid Charm" as indicated by the significant protocol genotype interaction. However, protocol x replicate or genotype x replicate interactions were not significant

.
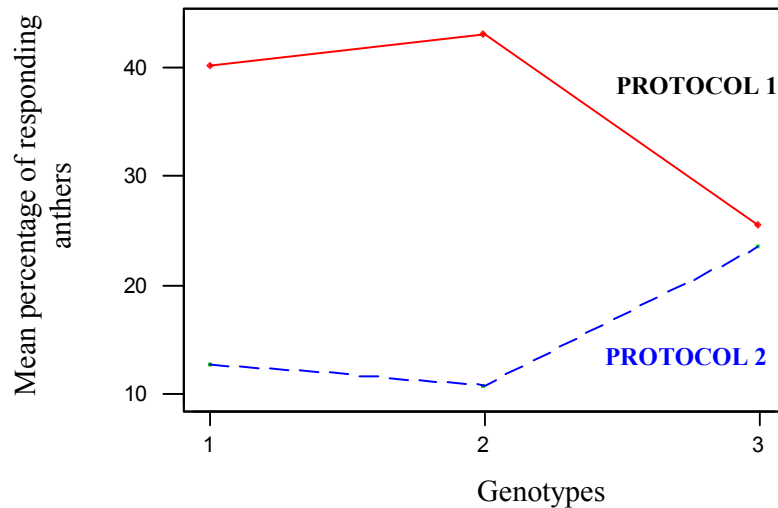
Figure 1.1. Interaction plot-showing three genotypes and their responses for the two protocols.

Genotype 1 = Snow Mountain
Genotype 2 = Cashmere
Genotype 3 = Candid Charm
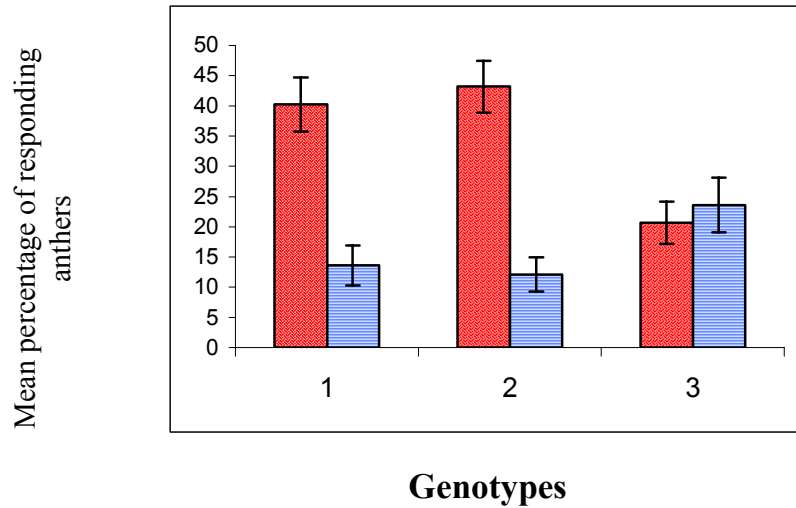
Figure 1.2. Anther culture response for the tested protocols.

I = Standard errors
Genotype 1 = Snow Mountain
Genotype 2 = Cashmere
Genotype 3 = Candid Charm

◆ Protocol 1

◆ Protocol 2

Post-culture observations revealed that with both protocols, induced anthers were swollen, embryo-like structures (ELS) emerged through the ruptures in the anther wall and generally there was no observable oozing out of microspores (Figure 1.3 A, B). With both protocols, induced anthers formed embryo-like structures (Figure 1.4 A, D) and callus (Figure 1.5 B, C, D). However, formation of callus was in a sporadic manner and they were of different morphological forms, e.g. loose friable (Figure 1.5 B), creamy (Figure 1.5 C) or compact nodular (Figure 1.5 D).
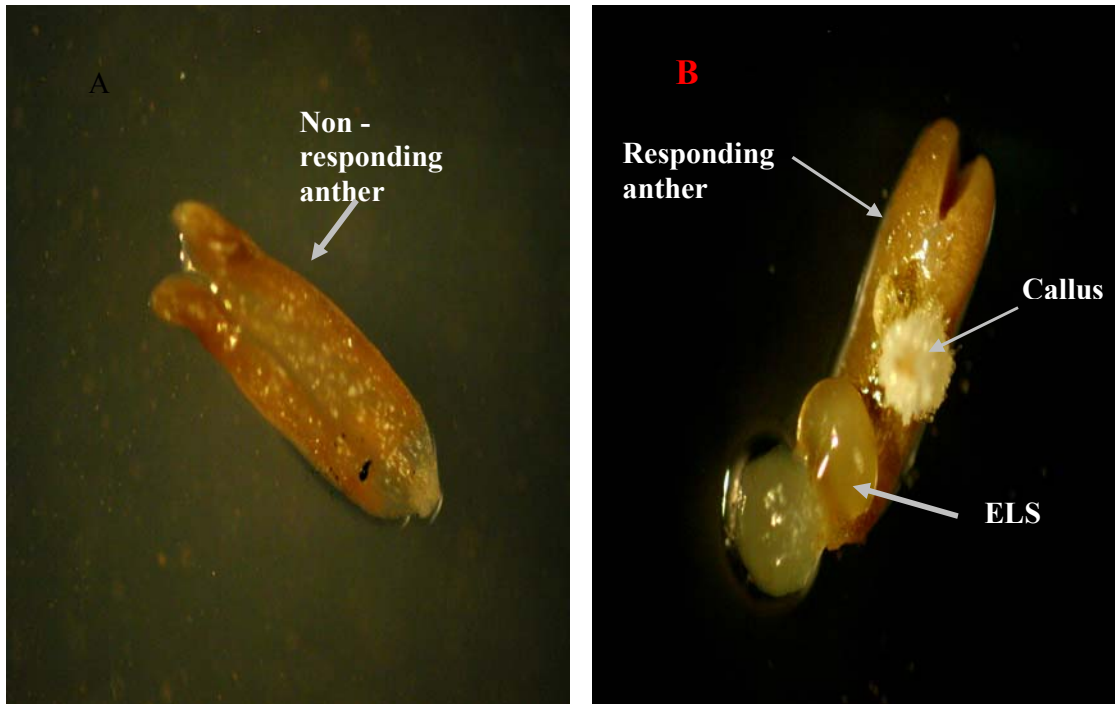
**Figure 1.3.** Responding and non-responding anthers.

      A. Non- responding anther (Snow Mountain, at five weeks with Protocol 2).
      B. Responding anther (Cashmere, at five weeks with Protocol 2), bearing ELS and callus.
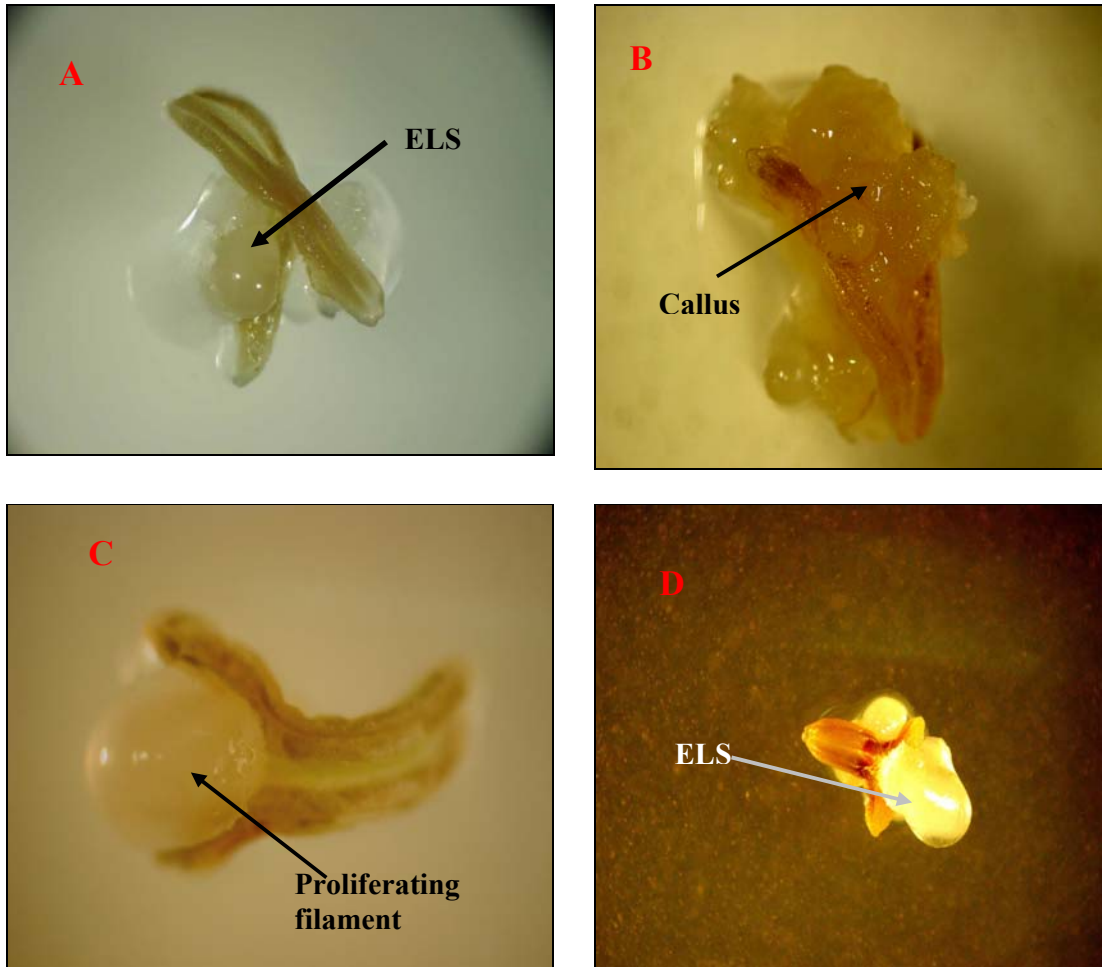
Figure 1.4. Various androgenic responses to Protocol 1.

    A. Responding Cashmere anther, at five weeks.
    B. Responding Snow Mountain anther, at five weeks.
    C. Responding Cashmere anther, at five weeks with remnants of filament.
    D. Responding Candid Charm anther, at five weeks.
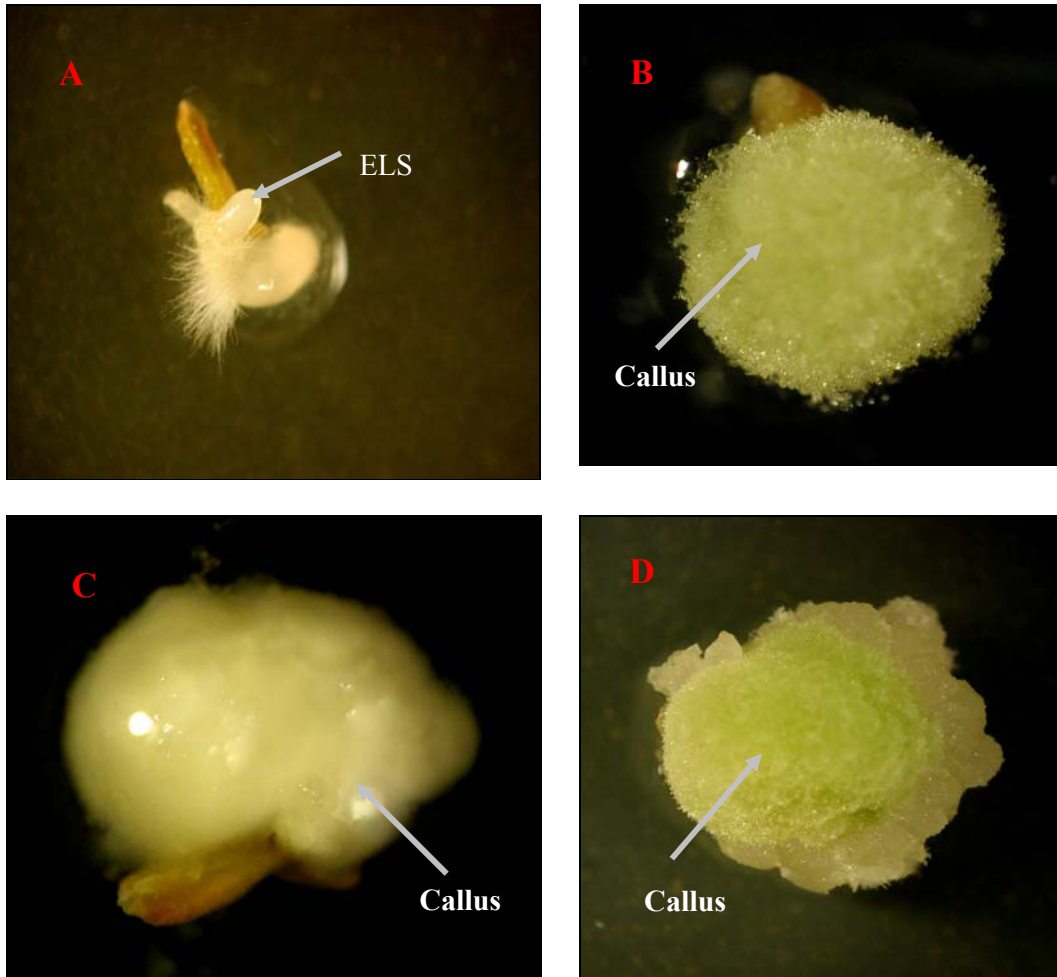
Figure 1.5. Various androgenic responses observed with Protocol 2.

- **A**. Snow mountain anther with embryo-like structures at five weeks.
- **B**. Cashmere anther with callus at five weeks (loose friable callus).
- **C.** Snow Mountain anther with callus at six weeks (creamy callus).
- **D.** Snow Mountain anther with callus at eight weeks (compact, nodular callus).

2. Sequential optimisation of an anther culture protocol for cauliflower for four cultural factors

2.1. Determination of optimum bud size

2.1.1. Experiment one–culture of anthers belonging to various bud size classes

There was a significant difference in the percentage of induced anthers from each of bud size classes. However, there were no significant genotype or genotype x treatment interaction Among the three classes, the percentage of induced anthers for Class-1 was significantly lower than for Classes-2 and 3 (Figure 2.1). Buds of this size class produced significantly fewer induced anthers, with a mean value of about 3%. Although there was no significant difference between the Class -2 and 3 for the induction, results indicated that the Class-2 flower buds were as marginally better (Figure 2.3). For Classes-2 and 3, mean values for the induced anthers were about 41 and 31% respectively.
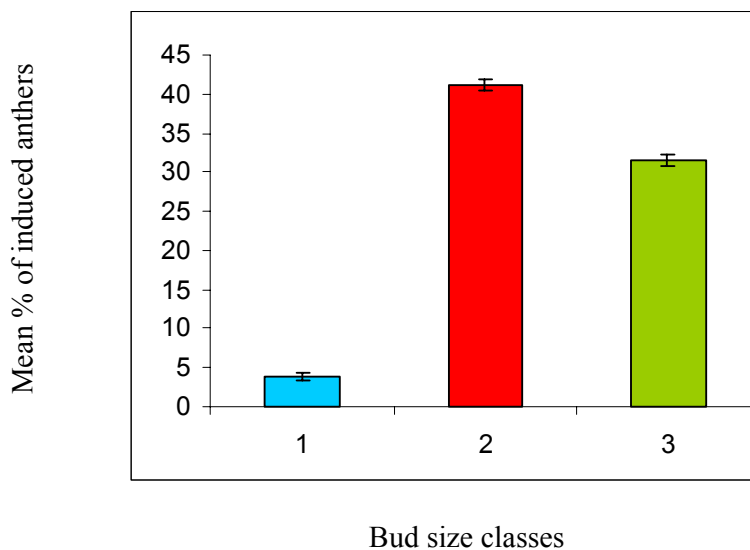
Bud size classes

Figure 2.1. Percentage of induced anthers for the three tested bud size classes (pooled results for three genotypes). I = Standard errors.
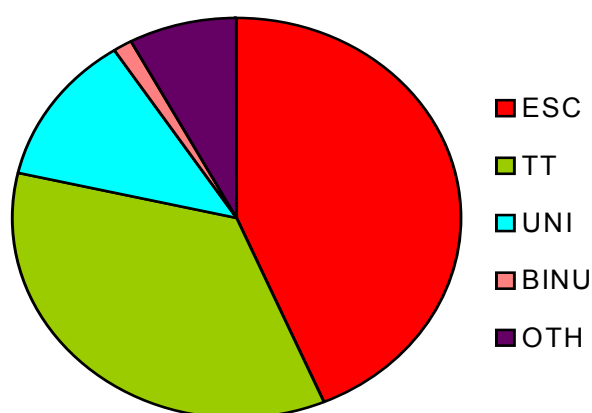
Class 1 = Less than 2 mm flower buds
Class 2 = 2-4 mm flower buds
Class 3 = Greater than 4 mm flower buds

2.1.2. Experiment two–cytological observation of various microspore developmental stages in various bud size classes

Microscopic examination revealed that the majority of microspores in Class-1 were at the early cell division and tetrad stages (Figure 2.2), while Class-2 and 3 flower buds consisted majority of microspores at the uninucleate and binucleated stages (Figure 2.3 and Figure 2.4). In Class-2, the proportion of uninucleate microspores is higher than the binucleated microspores and in Class-3; the proportion of binucleated microspores is higher than the uninucleate microspores. When the results of the two experiments were combined together, the indication was, that the most responsive flower buds for the tested genotypes were from Classes 2 and 3, which contained predominantly uninucleate and binucleated microspores. Furthermore, the results indicated that buds containing a higher proportion of uninucleate stage microspores respond marginally better, than the buds with higher proportion of binucleated stage microspores.
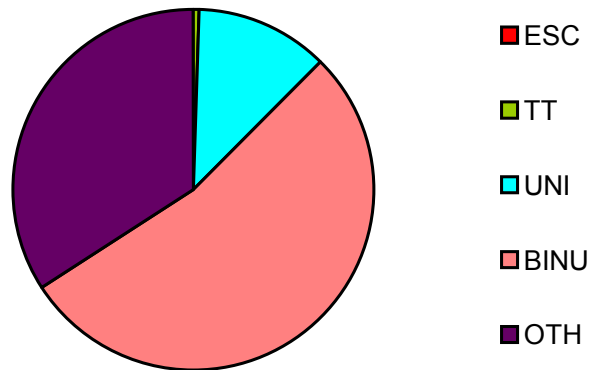

Figure 2.2. Distribution of different microspore developmental stages within the Class-1 (less than 2 mm) flower buds in all genotypes (pooled results).

ESC = Early stages of cell divisions
TT = Tetrads
UNI = Uninucleate stage
BINU = Binucleated stage
OTH = Other stages of cell divisions
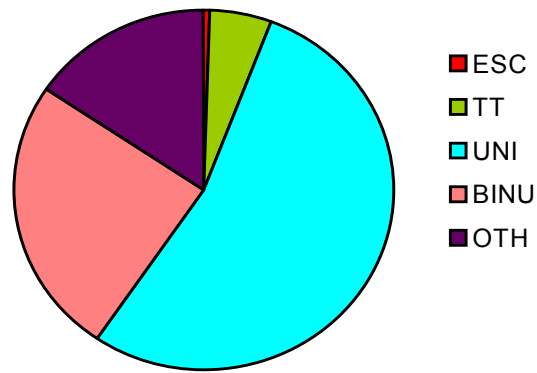
Figure 2.3. Distribution of different microspore developmental stages with in the Class-2 (2- 4 mm) flower buds in all genotypes (pooled results).

ESC = Early stages of cell divisions
TT = Tetrads
UNI = Uninucleate stage
BINU = Binucleate stage
OTH = Other stages of cell divisions

Figure 2.4. Distribution of different microspore developmental stages with in the Class-3 (greater than 4mm) flower buds in all genotypes (pooled results).

ESC = Early stages of cell divisions
TT = Tetrads
UNI = Uninucleate stage
BINU = Binucleated stage
OTH = Other stages of cell divisions

2.2. Factorial experiments with silver nitrate, sucrose and heat shock
2.2.1. Determination of effect of silver nitrate

Silver nitrate had a significant effect on the percentage of induced anthers in all three genotypes. However, "Snow Mountain" responded significantly better in the presence of silver nitrate than "Cashmere" or "Candid Charm", as indicated by the significant genotypic x treatment interaction (Figure 2.5). Additionally, except for the control treatment (in the absence of silver nitrate), there were no significant response differences to the other tested silver nitrate concentrations (7, 15 and 30 ppm). Although not significant, results indicated a possible inhibitory effect of silver nitrate for "Snow Mountain" at higher concentrations (Figure 2.5).
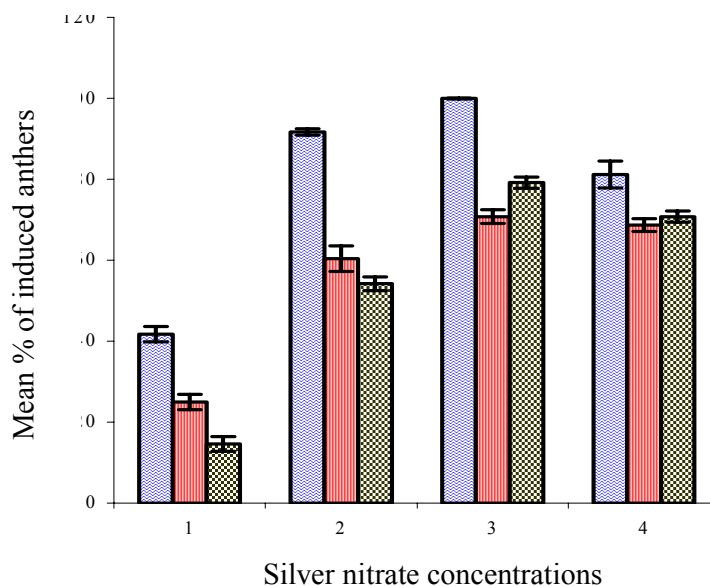
Figure 2.5. Anther culture response for the tested silver nitrate concentrations.
I = Standard errors

Concentration 1 = 0 ppm silver nitrate
Concentration 2 = 7 ppm silver nitrate
Concentration 3 =15 ppm silver nitrate
Concentration 4 = 30 ppm silver nitrate

■ Snow Mountain
■ Cashmere
■ Candid Charm

26

2.2.2. Determination of optimum sucrose concentration

The analysis of variance for the various concentrations of sucrose tested, indicated a significant treatment (P= 0.000) and a significant treatment x replicate interaction (P= 0.004). Among the treatments, the mean percentage of induced anthers for15% sucrose was significantly lower (Figure 2.6). Although there were no significant differences among the other treatments, results indicated that, 10% sucrose was marginally better. Thus, the overall results indicated that the sucrose concentration between 10 and 15% is more suitable for the tested genotypes (Figure 2.6).
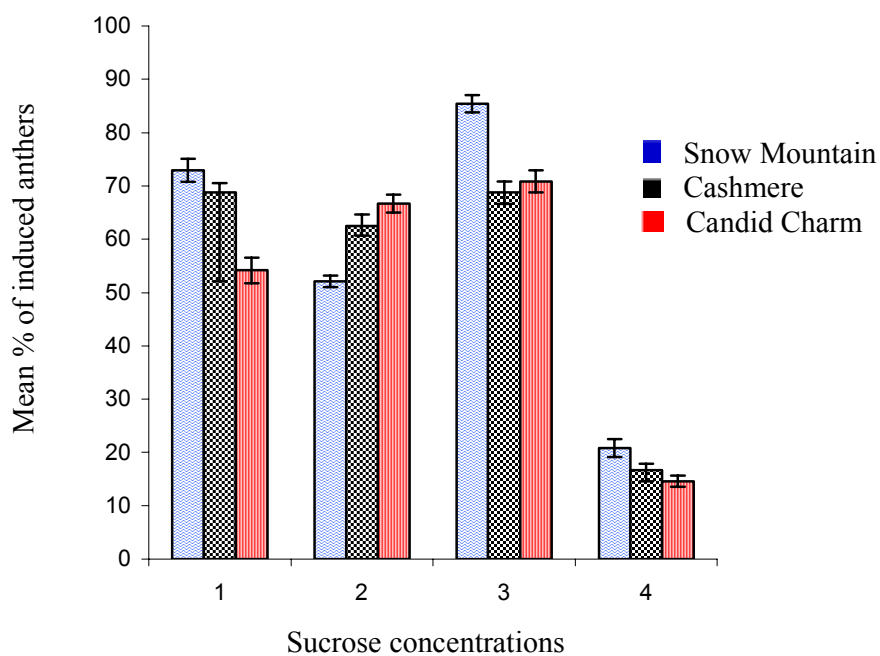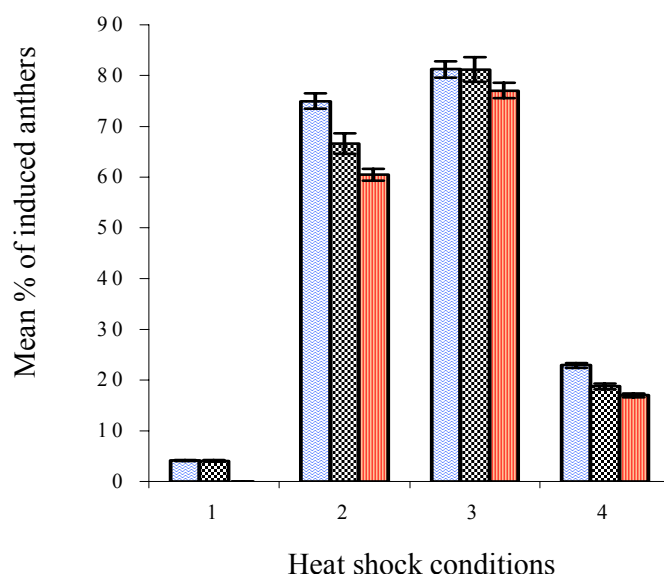

Figure 2.6 Anther culture response for the tested sucrose concentrations.
I = Standard errors

Concentration 1 = 2% sucrose
Concentration 2 = 6% sucrose
Concentration 3 = 10% sucrose
Concentration 4 = 15% sucrose

2.2.3. Determination of optimum heat shock duration

The analysis of variance for this experiment indicated a significant effect of treatments on the percentage of responding anthers. Among the treatments, T1 produced a significantly lower percentage of induced anthers. At this level "Candid Charm" was non-responsive. With this treatment, plated anthers were incubated at 25^0C continuously in the absence of heat shock. The other treatments, T2, T3 and T4 (all with varying heat shock conditions) yielded significantly higher percentages of responding anthers (the mean values were about 67, 80 and 20% respectively). Thus, results indicated the necessity of heat shock in obtaining a significantly higher numbers of responding anthers with the tested genotypes. Among the heat shock conditions, T4 was significantly different to T2 and T3 and contributed for a lower induction. Thus, the results indicated that the optimum heat shock condition as at 35^0C for 48 h hours and a possible inhibitory effect at more than 48 hours duration (Figure 2.7).

Figure 2.7. Anther culture response for the tested heat shock treatments.
        I = Standard errors

        Condition 1 = at 25^0C
        Condition 2 = at 35^0C for 24 h
        Condition 3 = at 35^0C for 48 h
   Condition 4 = at 35^0C 72 h

        ■ Snow Mountain
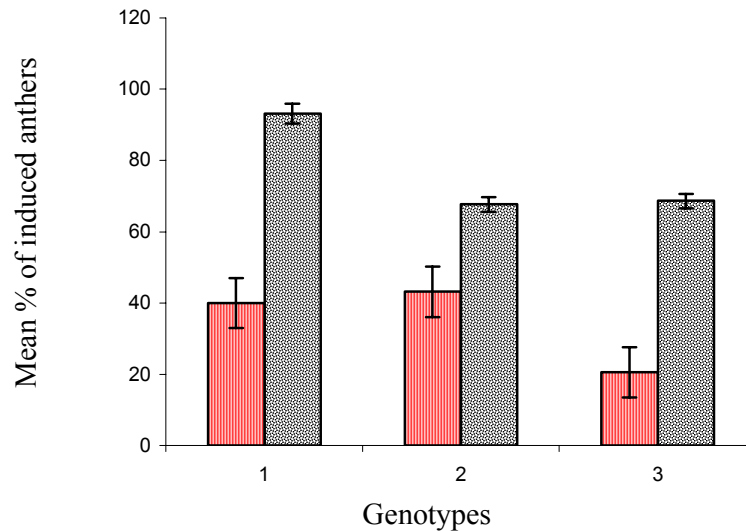   ■ Cashmere
        ■ Candid Charm

28

2.3.The effectiveness of the optimised protocol relative to the initial protocol (Protocol 1)

With the optimised protocol the majority of induced anthers formed several ELS, which were originated from entire anther body (Figure 2.8). They were all grouped and clumped together and had prevented the individual counting. Although callus formation was observed it was in a sporadic manner. Analysis of variance for the two protocols indicated that they were significantly different from each other in the percentage of induced anthers. The optimised protocol produced a higher percentage of induced anthers in all genotypes (Figure 2.9). Moreover, androgenic response of "Snow Mountain" to optimised protocol was significantly higher as indicated by the significant protocol x genotype interaction.
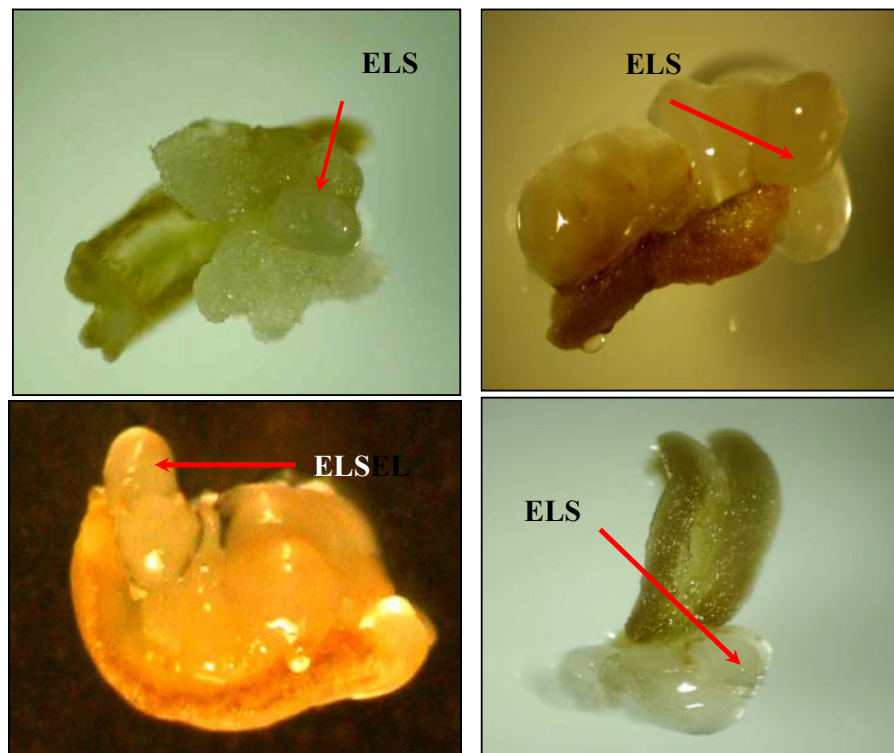
Figure 2.8. Anther culture responses for the optimised protocol (Snow Mountain anthers at five weeks indicating ELS formation throughout anther body).

Figure 2.9. Anther culture response for the initial (Protocol 1) and optimised protocols.
   I = Standard errors

   Genotype 1 = Snow Mountain
   Genotype 2 = Cashmere
   Genotype 3 = Candid Charm

   ▥ = Protocol 1 (initial protocol)
   ▨ = Optimised protocol

3. Regeneration and maturation of embryo like structures (ELS) and callus

When induced anthers were transferred to the tested regeneration media (MS and B5), plantlets were regenerated *via* various developmental pathways. On both culture media, initially developed nodular compact calli, further proliferated, and subsequently formed roots, and shoots *via* organogenesis (Figure 3.1). The other morphologic forms of calli induced, loose friable and creamy although proliferated remained as non-embryogenic without developing roots or shoots.

Induced embryo-like structures matured and regenerated into plantlets *via* several pathways (Figure 3.2). Majority of ELS during regeneration dedifferentiated into nodular callus and subsequently formed roots and shoots *via* organogenesis (callus pathway). Some ELS directly matured passing through various embryo developmental stages such as globular, torpedo (Figure 3.3) and eventually regenerated into plantlets (direct embryogenic pathway). Some ELS formed secondary embryos.
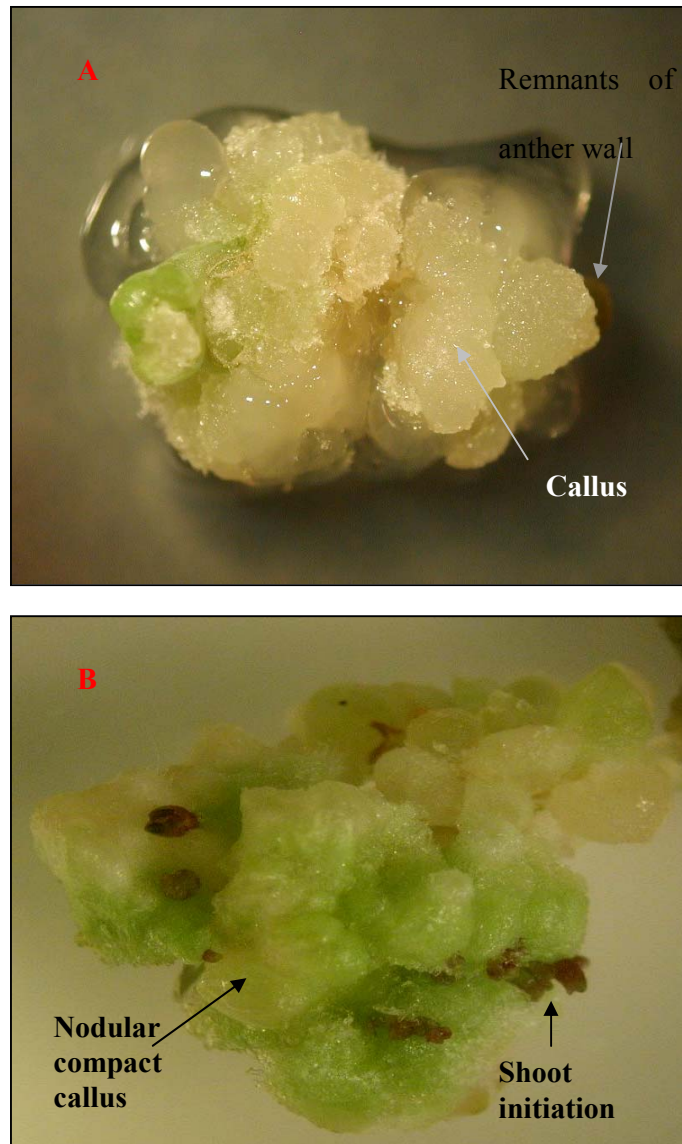
Figure 3.1. Callus formation and shoot initiation.

        A. Callus proliferated from Candid Charm induced anther (at four weeks on MS).
        B. Nodular callus with shoot initiation (Snow Mountain on MS at seven

These secondary embryos after several subculturing steps developed into plantlets. It was observed that many of the plantlets derived on B5 medium, were normal (with respect to leaf shape, general plant habit), healthy, and were relatively easier to harden-off while many developed on MS possessed abnormal leaf structures (Figure 3.4) and were comparatively harder to acclimatise. As statistical tests were difficult to perform with this type of data, it is unclear whether these observed differences were significant. In general, on both media, plant regeneration frequencies under aseptic conditions were relatively higher while hardening–off problems occurred when they were transplanted to soil.
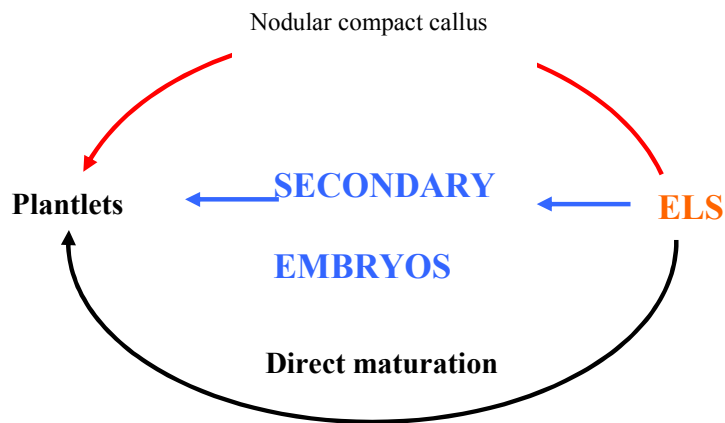
Figure 3.2. Various developmental pathways associated with ELS during their regeneration and maturation.

A
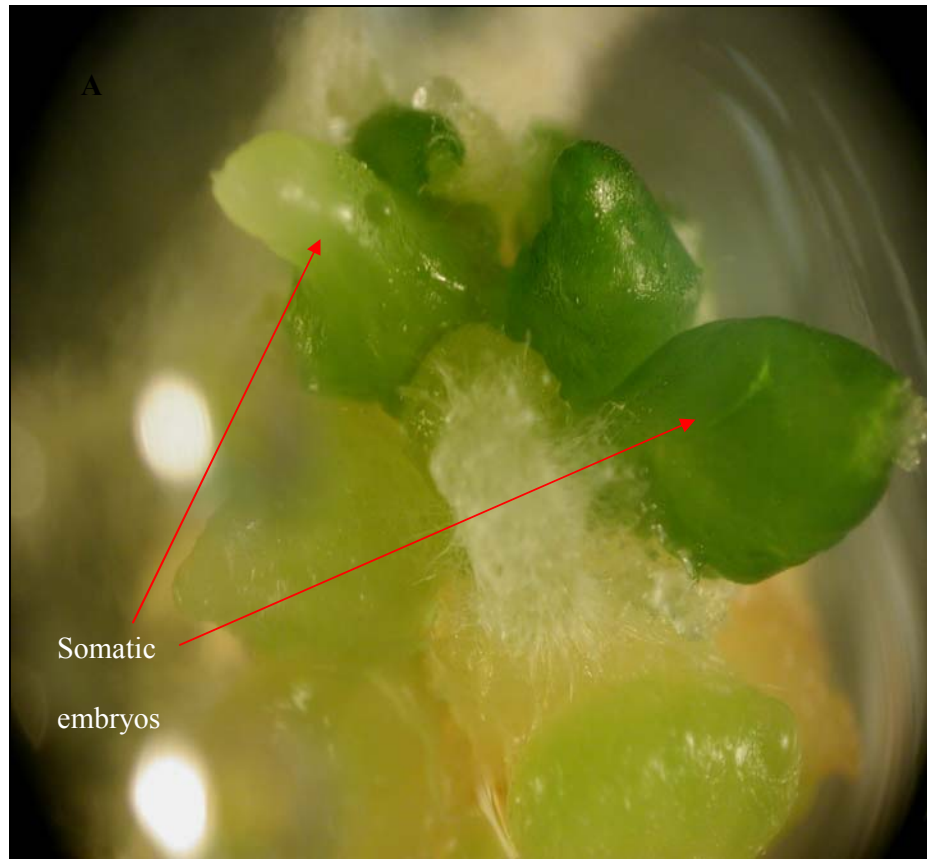
Somatic

embryos

Figure 3.4. Direct maturation of ELS (somatic embryos).
A. Snow Mountain at five weeks on B5.
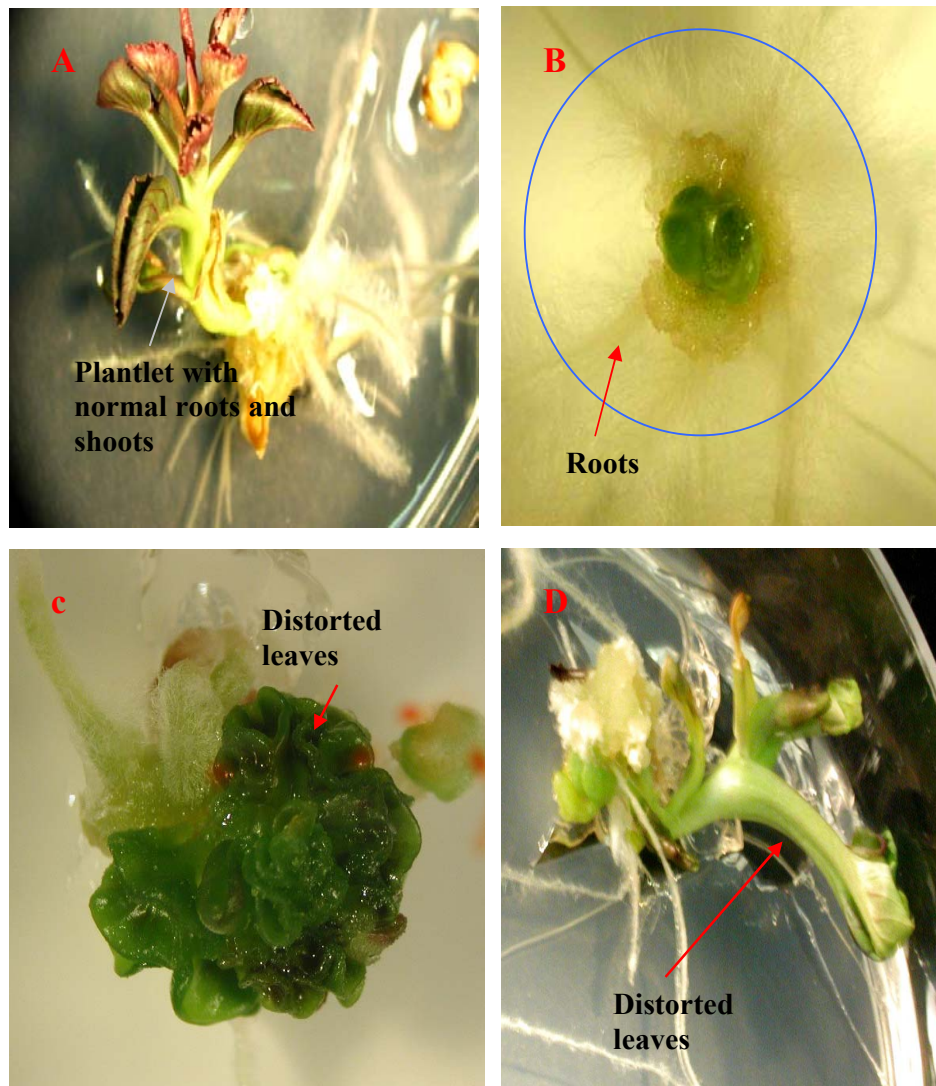
Figure 3.5. Development of plantlets *via* callus phase.

A. Snow Mountain plantlet on B5 with normal roots and shoots (at nine weeks).
B. Snow Mountain plantlet on B5 at its initial stages with roots (at seven weeks).
C. Candid Charm Plantlet originating on MS with distorted leaves (at seven weeks).
D. Cashmere plantlet originating on MS with distorted leaves (at nine weeks).

The results indicated a significant difference between the two media, in terms of the formation of callus and in roots/shoot development. Formation of calli was significantly higher on MS than on B5. In this context, there was no significant genotypic effect. The mean values for callus formation were on average 4 and 2 for six plated induced anthers on MS and B5 media respectively (Figure 3.6). Furthermore, in all three genotypes average size of the callus developed on MS was significantly lager than on B5 (Figure 3.7). The average values for size were 1 and 0.5 cm on MS and B5 respectively. Thus, for the formation of callus MS was superior to B5. But, in terms of root and shoot development, results indicated the opposite. On B5, root and shoot formation was significantly higher than on MS (Figure 3.8) in all genotypes. The mean values for root/shoot development were 0.6 and 2 for six induced anthers plated on MS and B5 respectively.
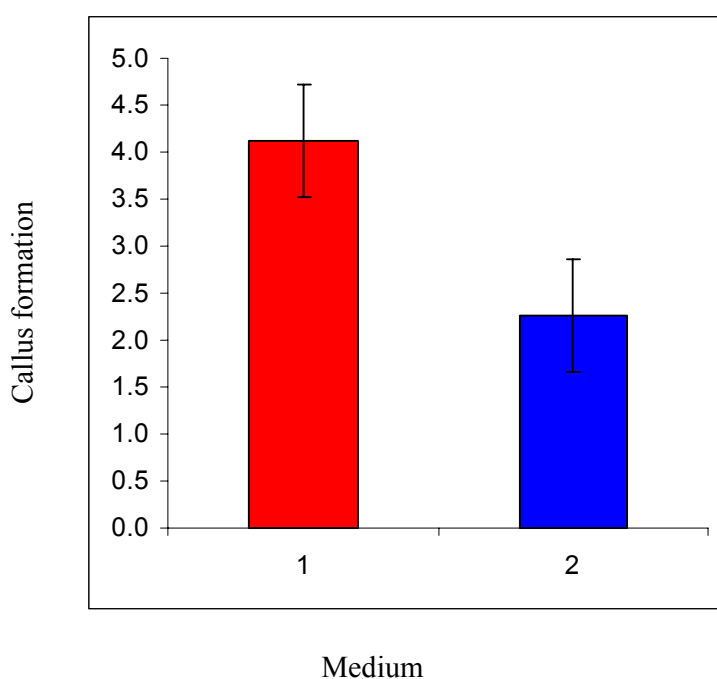
Medium

Figure 3.6. Influence of the culture medium in callus formation.
(Pooled results for three genotypes)
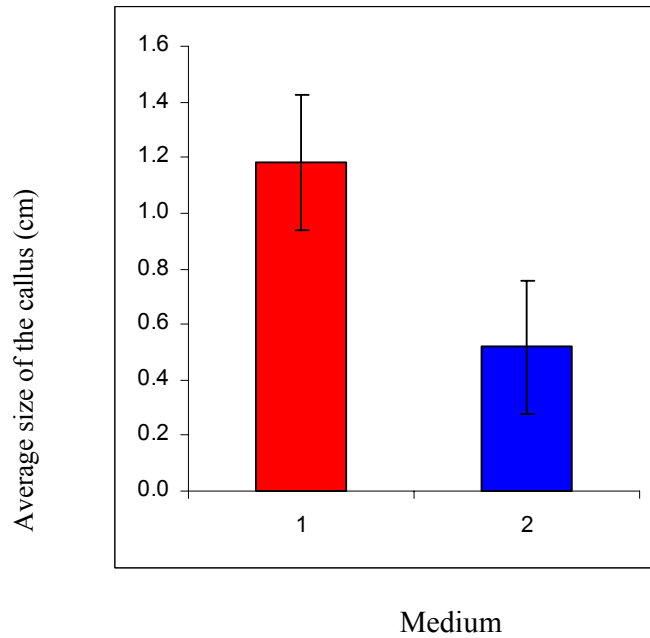
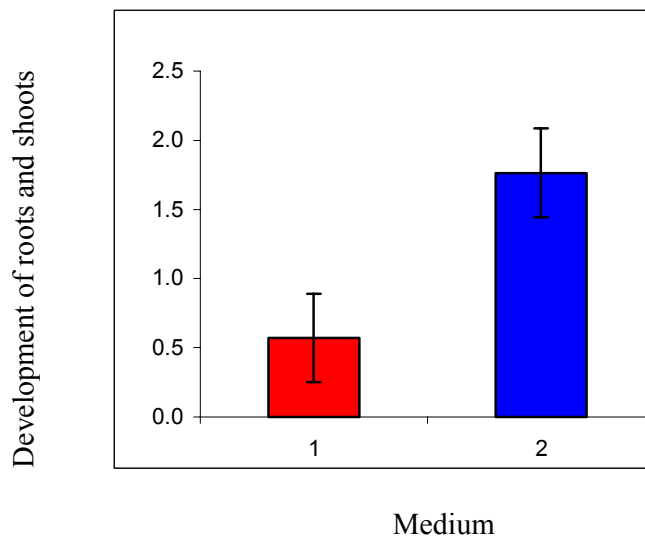I = Standard errors

MEDIUM 1 = MS.
MEDIUM 2 = B5.

Figure 3.7. Influence of the medium on callus size (pooled results for three genotypes).

I = Standard errors

MEDIUM 1 = MS.
MEDIUM 2 = B5.

Figure 3.8. Influence of the culture medium on root and shot development (pooled results for the three genotypes).
I = Standard errors

MEDIUM 1 = MS.
MEDIUM 2 = B5.

4. Ploidy level determination of anther derived regenerants for identification of haploids, diploids (Doubled haploids) and polyploids

4.1. Morphology

The 30 anther-derived regenerants observed in this study expressed obvious morphological differences. Until they reached their reproductive stage, these were mainly reflected by differences in leaf morphology (LM). It was possible to group the regenerant population into three distinct groups using LM (Table 4.1 and Figure 4.1). However, there were few plants that did not fit into either of these groups (4%). Most of the regenerants resembled their donors in terms of leaf morphology (40%). However, 23% plants possessed thin, long, filiform leaves or short-broader rounded leaves (33%). As plants matured these morphological differences became less distinct within the population. Although there were habit (branching) differences, was unfeasible to group the population using this character (variations were very low and only two regenerants showed different branching patterns).

Table 4.1. Different groups of leaf morphologies and their frequencies in the regenerant population.

GROUP	Description	Frequency
Group 1	Thin, long, filiform leaves	23%
Group 2	Short, broader, rounded leaves	33%
Group 3	Resembles donor plant leaf morphology (ellipsoidal)	40%
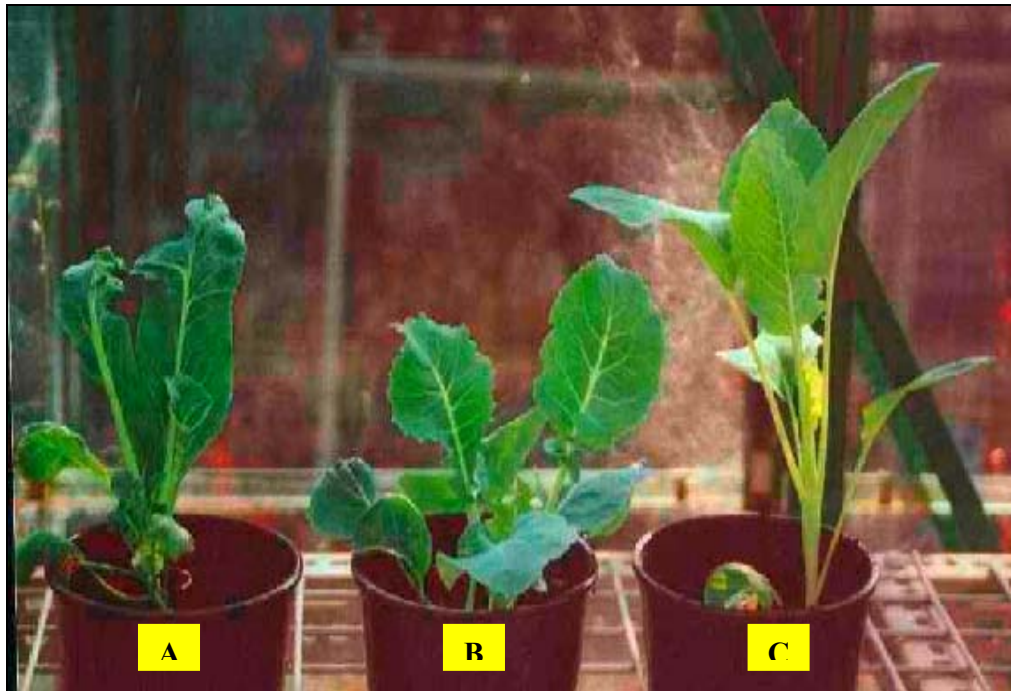
Figure 4.1. Anther derived regenerants with different leaf morphologies.

   A. Group 1 = Snow Mountain regenerant with thin, long, filiform leaves.
   B. Group 2 = Cashmere regenerant with short, broader, rounded   leaves.
   C. Group 3 = Snow Mountain regenerant resembling donor plant leaf morphology.

At their reproductive stage, regenerants exhibited flower colour and shape variations. White and yellow flowers were the two colours observed within the regenerants (Figure 4.2). Yellow flower colour frequency was high (90%) and resembled the flowers of donor plants. White colour frequency was low (10%). There were flower shape abnormalities among the regenerants. Some produced wrinkled flowers. All regenerants were fertile and there were no sterile plants. Under glasshouse conditions they produced seeds.

Figure 4.2. Flower colour variations observed in the regenerants.

        A. Snow Mountain regenerant with white flowers.
        B. Snow Mountain regenerant with yellow flowers (resembling donor plants).

4.2. DNA flow cytometry

DNA flow cytometric results confirmed the presence of diploids, tetraploids, aneuploids and chimeras. There were no confirmed haploids. Histograms generated for some of the regenerants appeared similar to the diploid histogram, which indicated that they might be doubled haploids or diploids derived from maternal tissue. The other generated histograms indicated tetraploids, aneuploids or chimeras. In all this study confirmed the presence of 12 diploids (60%), seven (35%) tetraploids and one chimera (15%) among the tested regenerants.

4.3. Molecular marker based identification

DNA amplifications were successfully obtained for all genotypes and for most of the regenerants, using the seven primers described by Kresovich *et al*. (1995). Table 4.2 and 4.3 shows the numbers of amplicons and their sizes obtained. However, SSR primers tested did not

produce polymorphisms between the genotypes used in this study. They either showed same banding pattern or, in some cases very faint bands different to others, which were not reproducible (Figure 4.3 and 4.4). This was the case with both the phase A and B of the experiment.

Table 4.2. PCR amplifications for Phase A genotypes.

PRIMER	POLYMORPHISM DETECTED [1]	NUMBER OF AMPLICONS	AMPLICON SIZE FROM THE PRESENT STUDY (bp)
B.N. 6A2	No	2	100, 120
B.n.16A	No	4	40, 170, 240, 300
B.n.18A1	No	3	130, 150, 300
B.n.20A	No	4	40, 75, 150, 300
B.n.9A	No	2	60, 240
B.n.25A	No*	-	-
B.n.6A3	No	2	40, 75

[1] between two parents (W, Y) and hybrid (H).
* reproducible PCR amplifications were not produced.

Table 4.3. PCR amplifications for Phase B genotypes (F1 hybrids and regenerants).

PRIMER	POLYMORPHISM DETECTED [11]	NUMBER OF AMPLICONS	AMPLICON SIZE FROM THE PRESENT STUDY (bp)
B.N. 6A2	No	1	90
B.n.16A	No	2	80, 200
B.n.18A1	No	3	150, 190, 200
B.n.20A	No	2	550, 800
B.n.9A	No	2	60, 300
B.n.25A	No*	-	-
B.n.6A3	No	1	240

[11] between F1 hybrids (Snow Mountain, Cashmere, Candid Charm) and anther derived regenerants.

  * reproducible PCR amplifications were not produced.
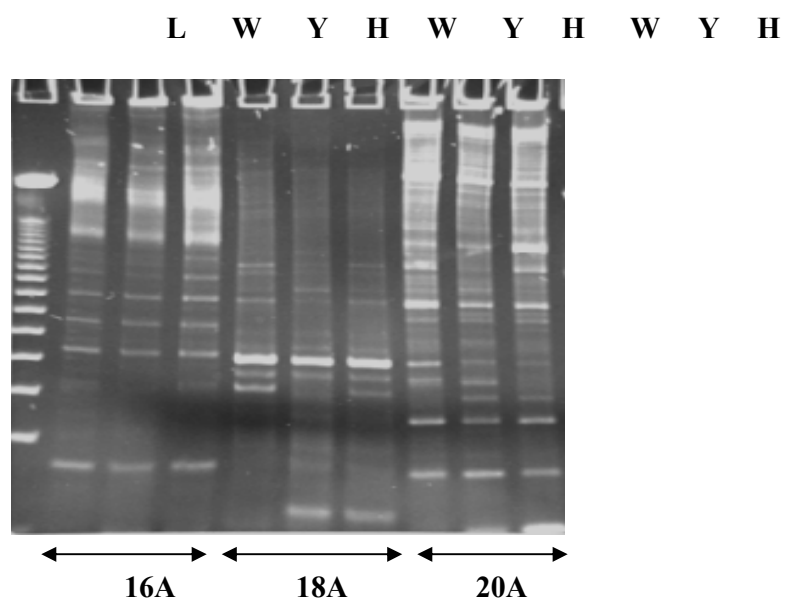
Figure 4.3 Segregation of SSR markers, B.n.16A, 18A1 and 20A in two parents (W, Y) and hybrid (H).   L = 50 bp ladder.

L   S   C   CC   R₁   R₂   R3   R₄

9A

Figure 4.4 Segregation of SSR marker B.n. 9A in three F1 hybrid individuals (Snow Mountain (S), Cashmere (C), Candid Charm (CC)) and anther derived regenerants (R$_1$, R$_2$, R$_3$, R$_4$). R$_1$ derived from Cashmere, R$_2$ and R$_3$ derived from Candid Charm and R$_4$ derived from Snow Mountain. L= 50 bp ladder.

DISCUSSION

The amenability to androgenesis was investigated *via* anther culture. Screening existing anther culture protocols on Australian varieties indicated their amenability to androgenesis. Since there were no previous investigations concerning the Australian varieties and this turned up to be the first, this discovery contributes positively to future studies on establishing DH technology in Australian cauliflower breeding programs.

For the induction of androgenesis, the genotypic variation observed in this study have been reported previously for many other plant species (Aiti *et al*., 1999; Guo and Pulli, 2000; Chaudhary *et al*., 2003; Zamani *et al*., 2003) and for *Brassica,* with almost all anther/microspore culture attempts (Dunwell *et al*., 1985; Lichter *et al*., 1989; Yang *et al*., 1992; Barro and Martin, 1999; Ferrie *et al*., 1999; Lionneton *et al*., 2001; Achar, 2002). For cauliflower, genotypic variation in androgenesis had previously reported by few authors (Ockendon, 1988; Fuller and Turton, 1990; Yang *et al*., 1992; Stipic and Campion, 1997).

The anther culture response is known to be under the influence of cytoplasmic (Malik *et al*., 2001) and nuclear-cytoplasmic interaction (Ekiz and Konzac, 1991). Thus the observed genotypic variation may be the result of these genetic regulation factors (Aiti *et al*., 1999). Additionally, genotypic differences may possibly be associated to their sensitivity differences to plant growth regulators (De Buyser and Henry, 1996). In this sense, it was suggested that genes involved in plant growth regulator metabolism or in their signal transduction pathways may play an important role (Aiti *et al*., 1999).

The genotypic variation in androgenesis may be effectively minimised by breeding (Aiti *et al*., 1999). In barley, a cross between responsive and non-responsive genotypes resulted in a hybrid with improved androgenic response (Ouedraogo *et al*., 1998). Similar results were found in cabbage where crosses between responding and non-responding genotypes followed by further backcrossing is recommended for converting a wide range of low responding genotypes into highly inductive genotypes (Rudolf *et al*., 1999). This approach is valuable as the lack of an efficient protocol for genotype-independent haploid induction had limited the use of the doubled haploid procedure in many plant-breeding programs (Rudolf *et al*., 1999). If this approach is successful for cauliflower, a cross between "Snow Mountain" and "Cashmere" may possibly result in a hybrid with improved androgenic response.

In the present study, significantly higher number of induced anthers was formed with the Protocol 1, where the medium was solid. This is in concurrence with the findings of Phippen and Ockendon (1990) and Stipic and Campion (1997), where significantly higher induction frequencies were achieved from cauliflower anthers cultured on solid media. However a previous study by Yang *et al*. (1992), on cauliflower anther culture discovered increased induction frequency on a liquid culture medium. Even in this study, "Cashmere" developed marginally higher number of induced anthers with Protocol 2 (Figure 2.2), where the medium was liquid. As indicated by this study, the suitability of solid or liquid medium may therefore possibly depend on the cauliflower genotypes under investigation.

The ELS formed in this study resembled somatic embryos, however various developmental stages such as globular, heart and torpedo were not observable at this stage (Figure 2.4 A, D and 2.5 A). From majority of ELS seedling like shoots grew out. This behavior was comparable to what has been reported by Lichter (1982) for *B. napus*. The majority of induced anthers (with both protocols) formed only 1-2 ELS (82%). Development of more than two ELS was rare and none of the anthers produced more than five ELS. However with Protocol 1, Keller *et al*. (1975)

observed 50% of anthers producing one embryoid and remainder producing 2-20 embryoids with *B. campestris* and with *B. napus,* 50% of anthers produced only one embryo while the rest producing few embryos (Keller and Armstrong, 1978). Fewer numbers of ELS formed per induced anther in this study possibly may have been due to the genotypes used. According to Stipic and Campion (1997), unlike the other species of *Brassica*, the majority of cauliflower genotypes are recalcitrant to anther culture.

As the initial induction of androgenesis *via* AC was at a lower frequency and of little practical benefit, the means to enhance the protocol was investigated. Among many potential factors, a few key factors were selected for the optimisation, which included: bud size, the level of sucrose, heat shock, an inhibitor of ethylene (silver nitrate). During the optimisation investigations, Class 2 and 3 flower buds that predominantly contained uninucleate and binucleated microspores were identified as the optimum bud size classes. This could be either due to both uninucleate and binucleated stages being reactive or may be due to the fraction of uninucleate microspores present in the larger flower buds. Although many previous studies on *Brassica* anther culture indicated only one reactive stage, the uninucleate stage (Thurling and Chay, 1984; Kott *et al.*, 1988) some have indicated two reactive stages, uninucleate and binucleated stages (Hansen and Svinnset, 1993). Additionally, few reports have indicated even tri-nucleated stage as a reactive stage in androgenesis (Kameya and Hinata, 1970). These differences may have been mainly accounted for by the different genotypes used.

It is not yet clear why androgenesis is restricted only to particular stage of microspore development. One suggestion is that, the late uninucleate to binucleated stages coincides with first pollen mitosis, the stage at which pollen is uncommitted either to androgenesis or to the normal mode of development, and therefore the application of stresses at this stage is capable of disturbing the normal development of such microspores thereby initiating androgenesis (Pechan and Smykal, 2001).

Based on the results of this experiment the length of the anther was utilised as an external tagging system for the selection of buds for the subsequent experiments. However, external tagging system based on length of anthers was reported as less accurate than the tedious microscopic examination technique as bud lengths may vary according to many factors such as growth conditions of the donor plants, genotypes and flowering cycle (first formed flowers vs. secondary formed flowers). To minimise these effects during this study, donor plants were grown under controlled environmental conditions.

Although few induced anthers were resulted in the absence of silver nitrate, from this study it was noted that the presences of silver nitrate in the medium is significantly enhancing the induction. Many former studies on *Brassica* anther culture have also indicated the beneficial effect of silver nitrate in androgenesis. In anther culture of Brussels sprouts, silver nitrate acted as a promoter of embryogenesis (Biddington *et al.,* 1988). In cabbage, the responses of cultured anthers, to the addition of silver nitrate was better over the controls while an increase in the concentration of silver nitrate had resulted in an increased production of haploid embryo yields (Achar, 2002).

The silver ion (Ag^+) is reported as a potent inhibitor of ethylene action (Ma *et al.*, 1998; Pullman *et al.*, 2003). Ethylene is known to influence many aspects of plant growth and development, including organogenesis and embryogenesis (Williams *et al.*, 1990; Pullman *et al.*, 2003). It is the believe that silver nitrate promotes embryogenesis by blocking the inhibitory effect of endogeneous ethylene on embryo production. Additionally, a study by Dunwell (1979) indicated a requirement of an optimum level of ethylene for embryo induction, above or lower than this

optimum the induction is inhibited. Thus, silver nitrate and other ethylene inhibitors may stimulate embryogenesis, in species having high endogenous levels of ethylene and may inhibit embryogenesis in species with lower endogenous ethylene concentrations (Biddington *et al*., 1988). This may be the reason for why effects of silver nitrate varies markedly between species or even with in the same species.

The results of this study indicated that 12% sucrose as more suitable for the tested genotypes. This compares favorably with many previous anthers culture studies on *Brassica,* where higher sucrose levels were being beneficial. For *B. oleracea* cv. *acephala,* 10% sucrose was found to be the optimum (Matsubayashi and Kuranuki, 1975). For *Brassica rapa*, it was 20% and for *B. campestris*, it was 10% (Keller *et al*., 1975). For *B. napus*, highest numbers of haploid embryos were obtained on 8% sucrose while for *B. juncea*, it was 17% (Lionneton *et al*., 2001). Although high sucrose levels were found to be beneficial for the early stages of androgenesis, lower sucrose levels were recommended for the latter stages (Dunwell and Thurling, 1985).

Sucrose contributes to the osmotic potential of the culture medium and act as a carbon source for cell growth and development (Kang *et al*., 2003). Thus, the concentration of sucrose would, therefore change during the culture process and the osmotic potential accordingly as well. The significantly low percentage of anthers induced with 15% sucrose could possibly have been due to the unsuitable osmotic potential generated at this level, creating unsuitable conditions for the developing microspores.

From this study it was evident that for the tested cauliflower genotypes heat shock is essential for androgenesis and most suitable condition discovered was at 35^0C for 48 h. Heat shock pretreatment appears to be the most important factor in *Brassica* anther culture where 30-35^{0}C was often employed. However, the optimal level required may vary with the genotypes. In cabbage, the maximum embryo yields were obtained with heat treatment of 24 h at 35^{0}C (Achar, 2002). For Brussels sprouts, it was 16 h at 35^{0}C (Ockendon, 1984). For broccoli, it was found to be 48 h at 35^{0}C. (Keller and Armstrong, 1983). The results of this experiment indicated a reduction in the percentage of induced anthers with increased duration of heat shock (three days at 35^{0}C). Similarly, in cabbage, embryo formation was completely inhibited with the treatment of 35^{0}C for six days (Achar, 2002). Treatment of 30^{0}C for 14 days had produced zero embryos in Brussels sprouts and broccoli anther cultures (Keller and Armstrong, 1983). This may have been possibly due to the loss of microspore viability with longer durations.

All tested genotypes formed significantly higher percentage of induced anthers with the optimised than with the initial protocol. Although a number of factors were responsible for this increased response (Table 3.3), the contribution of silver nitrate may be the most significant. However, unless a factorial experiment is performed this observation may not be fully substantiated. Throughout these experiments obtaining sufficient numbers of flower buds with the appropriate microspore developmental stages was problematic (due to asynchronicity of flowering of three genotypes). Given the large numbers of anthers required for such a factorial experiment, where all cultural factors are considered together the execution of such an experiment would have been extremely difficult.

Among the three regeneration pathways observed in this study (callus, direct maturation, and secondary embryogenesis), callus and direct maturation pathways were common whereas, secondary enbryogenesis was rare. However, secondary embryogenesis has been previously reported for *B. napus* (Cegielaka-Taras *et al*., 2002) and for some other plant species such as wheat (Hofer, 2003). Secondary embryogenesis is the proliferation of somatic embryos from each other and in some studies this phenomenon has been referred to as repetitive somatic

embryogenesis (Little *et al*., 2000). Although plantlets could be achieved from secondary embryos it required two or more subculture stages. Similarly for *B. napus*, regeneration *via* secondary embryos was reported as time and labor consuming process (Cegielska-Taras *et al*., 2002).

Interestingly, there was no significant genotypic effect indicated in this study. However, previous regeneration studies on *Brassica* and on other plant species (e.g. barley, soybean) indicated regeneration/maturation capacity as dependent on genotype and thus is under genetic control (Romeijn and Lammeren, 1999; Zamani *et al*., 2000). According to Zaki and Dickinson (1995), genotypic specificity in regeneration and maturation may occur not that different genotypes require different regeneration or maturation conditions, but due to different kinetics of cell division associated with different genotypes. Furthermore study did not indicate a direct relationship between the induction and regeneration capacity. For example, "Snow Mountain" had the highest induction capacity but had moderate regeneration capacity. Thus the results support the hypothesis, induction and regeneration processes are under separate genetic control (Santos *et al*., 1997; Komatsuda *et al*., 1989).

Development of plantlets on both MS and B5 is in accord with previous studies concerning *Brassica* where these two media were effective for regeneration and maturation (Duijs *et al*., 1992; Rudolf *et al*., 1999). However, many of the previous studies concerning this species have not been evaluated between these two media and had suggested either of these media as appropriate for regeneration/maturation. In this study, callus proliferation was significantly higher on MS while root and shoot development was significantly better on B5. On MS, induced calli further proliferated while majority of ELS reverted to the callus phase. On B5 majority of ELS matured directly and formed roots and shoots. With the present study why results indicated B5 as inferior to MS, in the formation of callus may underlined by this observation.

Regeneration *via* the callus pathway has some serious limitations of which main limitation would be the possible somaclonal variation that leads to the formation of chimeras and higher ploidy levels in the regenerant population, which are of little practical value. Thus, with the results B5 may be the most suitable regeneration/maturation medium for the tested genotypes. However on B5, on average 2 out of six plated ELS matured and formed root and shoots, which is a lower frequency. In rye and rice, decreased regeneration capacities were accounted for by the occurrence of mutations during the tissue culture process (Guo and Pulli, 2000; Kahawata *et al*., 1992). Experienced low regeneration frequency in this study may possibly be due to the genotypes used and therefore this aspect (regeneration and maturation process) in cauliflower anther culture warrants further future investigations.

Morphology, DNA flow cytometry and molecular-marker based identification methods were employed to analyse anther-derived regenerants for their ploidy level. Root tip mitosis chromosome counts were not employed in this study as the technique was disruptive and was tedious to perform with cauliflower where the chromosomes were small. The technique of GCL measurements was also time consuming and had produced overlapping values for haploid and diploid plants thus was, not selected either.

The morphology of anther-derived cauliflower plants was quite different to that of their diploid donor plants. Morphological differences in anther-derived regenerants have been previously reported for many other plant species and for Brassica (Keller *et al*., 1975; Chalyk and Chebotar, 2000; Kato, 2002). In the present study, morphology alone did not offer sufficient information, to precisely identify PL, but helped to group regenerants into three distinct morphological classes indicating the possible presence of three different ploidy levels within the regenerant population.

However, grouping of regenerants based on their morphological variations helped to obtain a representative sample with fewer numbers of individuals for DNA flow cytometric studies.

Based on DNA flow cytometry, there was no indication for the presence of haploids. Instead, it confirmed the presence of diploids, tetraploids, aneuploids and a chimera. The absence of haploids in an anther-derived population was not unique to this study, but has also been reported for *B. campestris* (Keller *et al*., 1975) and *B. napus* (Keller *et al*., 1978). In some instances, even though haploids were detected, frequencies were very low (Ockendon, 1988; Stipic and Campion, 1997; Wang *et al.,* 1999). Therefore, the population of 20 regenerants tested may not have been sufficient to permit the detection of haploids in this study. Gosal *et al*. (1988) have reported, the identification of haploid embryos in the early stages of corn anther cultures and complete absence of haploid cells in an old anther-derived callus cultures. This suggests that a lengthy culture period may results in spontaneous diplodisation of the haploid cells. This could be another reason for the failure to detect haploids among fully developed plants.

There are many possible reasons for the presence of higher ploidy levels. One explanation is endoreduplication and (or) nuclear fusion (Sunderland *et al*., 1974). Another explanation is that triploids arose by fusion of haploid and diploid nuclei early during pollen embryogenesis (Ockendon, 1988). Whichever process is occurring, the final ploidy compositions of anther-derived regenerants were reported to be species and genotype specific (Wang *et al*., 1999; Farnham, 1998). It is not yet known why some species and genotypes produce more haploids whereas others produce majority of diploids or polyploids. The stage of the pollen development at the time of culture may also affect subsequent ploidy levels of the regenerants. For example, culturing pollen at uninucleate stage resulted in the production of more haploids while pollen at binucleated stage resulted in more diploids and other higher ploidy levels (Sunderland *et al*., 1974; Keller *et al*., 1975).

The occurrence of spontaneously doubled haploids has been well documented in Brassica, with up to 10-26% spontaneous diploids being observed with some of the genotypes (Chen *et al*., 1994). In anther-derived Brussels sprouts, half of the regenerants were haploids and the other were diploids, with polyploids being only 2-9% (Ockendon *et al*., 1993). In cabbage, there were 12% haploids, 55% diploids and 32% tetraploids. In the present study, DNA flow cytometric method confirmed the presence of 55% diploids, which was quite a high frequency. Previous anther culture studies on cauliflower reported 41% (Ockendon, 1988), 26% (Wang *et al*., 1999) and 79% (Stipic *et al*., 1997) spontaneous diploids.

Although diploids were identified in this study there was still doubt as to whether these diploids originated from haploid tissue. Although there was evidence to support the haploid (pollen) origin, there was no certainty to their haploid origin. These plants may have two different origins, either from microspores as haploid cells or somatic cells from anther tissue. The DNA flow cytometric method was not suitable for discriminating between diploids of haploid origin (pollen) from diploids originate from maternal tissue (anther wall, connective tissue).

The molecular marker–based technique failed to generate polymorphisms between the lines used. Thus even though primers were polymorphic for *B. napus* they were not so for cauliflower. Ideally, SSRs designed from cauliflower should have been used for this study. However at the time of this study, there were no published reports on SSR loci isolated from cauliflower and the isolation of SSR loci from a species involves a great deal of time and effort, which is beyond the scope of this project. SSRs have been used successfully to determinate heterozygous and homozygous nature of regenerants from anther cultures in other plant species (2001; Murantly *et al*., 2002; Chani *et al*., 2000). Additionally, in the context of plant breeding, SSRs have

numerous other applications such as gene tagging and genetic mapping. Therefore, the isolation of SSR loci from cauliflowers would be of tremendous value to future plant breeding and tissue culture research.

Finally in all, this study investigated for the first time the prospect of developing DH, concerning the Australian varieties of cauliflower and had discovered AC as the most suitable technique. With the optimisation of a selected AC protocol study had established an anther culture protocol that may consider as an efficient. Investigations on further development and maturation of ELS were carried out and plantlets successfully developed were analysed for their ploidy levels.

TECHNOLOGY TRANSFER

The information obtained from this project was communicated in a number ways. Firstly, Henderson Seeds were updated on the progress of the project as necessary, especially when a major milestone was completed. Secondly, the Ph.D. student on this project, Ms. Ruchira Jayasinghe presented her results as a seminar at the Seventh Annual Scientific Symposium of the Joint Centre for Crop Improvement, which was held in Rutherglen, Australia, in November 2000. The Symposium was attended by over 80 participants from Agriculture Victoria, The University of Melbourne, Monash University and RMIT University. She is currently preparing a manuscript for publication in the Australian Journal of Botany entitled "Dihaploid plant development in Australian varieties of cauliflower: Effect of silver nitrate and heat shock". Finally, electronic copies of the Ph.D. thesis is available on request either from Dr. Eddie Pang (eddie.pang@rmit.edu.au) or Horticulture Australia.

RECOMMENDATIONS

A viable anther culture protocol has been developed for cauliflower. This protocol may be extended to other *Brassica* crops, and indeed several protocols are already available for overseas varieties of Broccoli. As the equipment necessary for the implementation of these protocols are usually not readily available to plant breeders, we suggest that interested *Brassica* breeders/researchers approach RMIT in the first instance, where we will provide a service for producing doubled haploids for their programs. Additionally, we are happy to provide free training for breeders who possess, or are able to access the necessary equipment for anther culture.

Further research should be undertaken on other means of generating doubled haploids, for example isolated microspore culture or gynogenesis. Additionally, methods for the confirmation of ploidy levels, especially those based on PCR technology should be explored.

ACKNOWLEDGEMENTS

BIBLIOGRAPHY

Achar, P. N. (2002). A study of factors affecting embryo yields from anther culture of cabbage. *Plant Cell Tissue Organ Culture.* **69**(2): 183-188.

Aiti, F. J., Benlhabib, O., Sharma, H. C., Jafari, S. E., and Hadrami, I. E. (1999). Genotypic variation in anther culture and effect of ovary co-culture in durum wheat. *Plant Cell Tissue and Organ Culture.* **59**: 71-76.

Baenziger, S., (1996). Reflections on doubled haploids in plant breeding. In S. M. Jain, S. K. Sopory, and R. E. Veilleux (eds), *In vitro* haploid production in higher plants, Vol1, pp. 35-48. Kluwer Acad. Publ. Dordrecht.

Bansal, Thiagarajah, Stringam and Tewari. (1999). Inheritance of partial resistance of race 2 of *Albugo candida* in canola-quality mustard (*Brassica juncea*) and its role in resistance breeding. *Plant Pathology*. **48**(6): 817-822.

Barro, F., and Martin, A. (1999). Response of different genotypes of *Brassica carinata* to microspores. *Plant Breeding*. **118**(1): 79-81.

Barro, F., Fernandz-Escobar, J., De La Vega, M., and Martin, A. (2001). Doubled haploid lines of *Brassica carinata* with modified erucic acid content through mutagenesis by EMS treatment of isolated microspores. *Plant Breeding.* **120**(3): 262-264.

Betty, M., Finch-Savage, W. E., King, G. J., and Lynn, J. R. (2000). Quantitative genetic analysis of seed vigor and pre-emergence seedling growth traits in *Brassica Oleracea. New Phytologist*. **148**(2): 277-286.

Bhalla, P. L., and Weed, N. D. (1999). *In vitro* propagation of cauliflower, *Brassica oleracea* var. *botrytis* for hybrid seed production. *Plant Cell Tissue Organ Culture.* **56**: 89-95.

Biddington, N. L., Sutherland, R. A., and Robinson, H. T. (1988). Silver nitrate increases embryo production in anther culture of Brussels sprouts. *Annals of Botany.* **62**: 181-185.

Cegielaka-Taras, T., Tykarska, T., Szala, L., Kuras, M., and Krzymanski, J. (2002). Direct plant regeneration from microspore derived embryos of winter oilseed rape *Brassica napus* L. ssp. *oleifera* (DC.) Metzger. *Euphytica.* **124**: 341-347.

Chalyk, S. T., and Chebotar, O. D. (2000). Regular segregation of four recessive marker genes among maternal haploids in maize. *Plant Breeding.* **119**(4): 363-364.

Chani, E., Veilleux, R. E., Medina, T. B. (2000). Improved androgenesis of interspecific potato and efficiency of SSR markers to identify homozygous regenerants. *Plant Cell Tissue Organ Culture.* **60**(2): 101-112.

Chaudhary, H. K., Dhaliwal, I., Singh, S., and Sethi, G. S. (2003). Genetics of androgenesis in winter and spring wheat genotypes. *Euphytica.* **132**: 311-319.

Chekurov, V. M., and Razmakhnin, E. P. (1999). Effect of inbreeding and growth regulators on the *in vitro* androgenesis of wheatgrass, *Agropyron glaucum. Plant Breeding* **118**(6): 571-573.

Chen J. L., Beversdorf, W. D. (1992). Production of spontaneous diploid lines from isolated microspores following cryopresservation in spring rapeseed (*Brassica napus* L.). *Plant Breeding*. **108**: 324-327.

Chen, Y., Kenaschuk, E., and Deribneki, P. (2001). Inheritance of rust resistance genes and molecular markers in microspores derived populations of flax. *Plant Breeding*. **120**(1): 82-84.

De Buyser, J., and Henry, Y. (1996). Analyse genetique de I, aptitude a I androgenese chez le ble tender. In: Petitprez (ed) Biotechnologie Vegetales " haplolodisation" (pp 98-101.) Centre National d ' Enseignement a Distance, Institut de Rennes, Toulouse.

Duijs, J. G., Voorrips, D. L., Visser, and Custers, J. B. M. (1992). Microsore culture is successful in most crop types of *Brassica oleracea* L. *Euphytica*. **60**; 45-55.

Dunwell, J. M. (1979). Anther culture in *Nicotiana tabacum*: The role of the culture vessel atmosphere in pollen embryo induction and growth. *J. Exp. Bot*. **30**: 419-428.

Dunwell, J. M. and Thurling, N. (1985). Role of sucrose in microspore embryo production in *Brassica napus* ssp. *oleifera*. *J Exp Bot*. **36**: 1478-1491.

Dunwell, J.M., Cornish, M., and De Courcel, A.G. L. (1985). Influence of genotype, plant growth temperature and anther incubation temperature on microspore embryo production in *Brassica napus* ssp. *olerifera*. *J Exp Bot*. **36**: 679-689.

Ekiz, A., and Konzak, C. (1991). Nuclear and cytoplasmic control of anther culture response in wheat: ii. Common wheat x alloplasmic lines. *Crop Sci*. **31**: 1427-1431.

FAO Statistical Database. 2002. http://apps.fao.org

Farnham, M. W. (1998). Doubled haploid broccoli production using anther culture; effect of anther source and seed set characteristics of derived lines. *J. Amer Soc. Hort. Sci*. **123** (1): 73-77.

Ferri,e A. M. R., Taylor, D.C., Mackenzie, S. L., and Keller, W.A. (1999). Microspore embryogenesis of high *sn*$^{-2}$ erucic acid *Brassica oleracea* germplasm. *Plant Cell Tissue and Organ Culture*. **57**: 79-84.

Foroughi-Wehr, B., and Wenzel, G. (1990). Recurrent selection alternating with haploid steps-a rapid breeding procedure for combining agronomic traits in inbreeders. *Theor. Appl. Genet*. **80**: 564-568.

Fuller, M. P., and Turton, S. (1990). Anther culture of winter heading cauliflower. *Acta Hort*. **280** 329-331.

Gosal, S. S., and Bajaj, Y. P. S. (1988). Pollen embryogenesis and chromosomal variations in anther culture of three food legumes-*Cicer arietinum*, *Pisum sativum* and *Vigna mungo* . *Sabrao Journal*. **20**(1): 51-58.

Guo, Y. D., Pulli, S. (2000). Isolated microspore culture and plant regeneration in rye (*Secale cereale* L.). *Plant Cell Reports*. **19**: 875-880.

Guzy-Wrobelska, J., and Szarejko, I. (2003). Molecular and agronomic evaluation of wheat doubled haploid lines obtained through maize pollination and anther culture methods. *Plant Breeding*. **122**(4): 305-313.

Hansen, M. and Svinnset, K. (1993). Microspore culture of swede (*Brassica napus* ssp. *rapifera*) and the effects of conditioned media. *Plant Cell Rep*. **12**: 496-500.

Hofer, M., and Grafe, C. (2003). Induction of doubled haploids in sweet cherry (*Prunus avium* L.). *Euphytica*. **130**(2): 191-197.

Immonen, S., and Anttila, H. (1999). Cold pretreatment to enhance green plant regeneration from rye anther culture. *Plant Cell Tissue Organ Culture* .**57**(2):121-127.

Kameya, T., and Hinata, H (1970). Induction of haploid plant from pollen grains of *Brassica Jpn. J. Breed*. **20**: 82-87.

Kang, T. J., Yang, M. S., and Deckard, E. L. (2003). The effect of osmotic potential on anther culture in spring wheat (*Triticum aestivum*). *Plant Cell Tissue and Organ Culture*. **75**: 35-40.

Kato, A. (2002). Chromosome doubling of haploid maize seedlings using nitrous oxide gas at the flower primordial stage. *Plant Breeding.* **121**(5): 370-377.

Kawata, M., Harada, S., Antonio, B., Onono, K. (1992). Protoclonal variation of plant regeneration in rice. *Plant Cell Tissue Organ Culture* **28**: 1-10.

Keller, W. A. and Armstrong, K. C. (1983). Production of haploids *via* anther culture in *Brassica oleracea* var. i*talica. Euphytica.* **32**: 151-159.

Keller, W. A., and Armstrong, K. C. (1978). High frequency production of microspore derived plants from *Brassica napus* anther cultures. *Z. Pflanzenzuchtg.* **80**: 100- 108.

Keller, W. A., Rajpathy, T., and Lacapre, L. (1975). *In vitro* production of plants from pollen in *Brassica compestris. Can. J. Genet. Cytol.* **17**: 655- 666.

Keller, W. A., Rajpathy, T., and Lacapre, L. (1975). *In vitro* production of plants from pollen in *Brassica compestris. Can. J. Genet. Cytol.* **17**: 655- 666.

Komatsuda, T., Enomoto, S., and Nakajima, K. (1989). Genetics of callus proliferation and shoot differentiation in barley. *J. Hered.* **80**: 345-350.

Kott, L. S., Polsoni, L and Beversdorf, W. D. (1988). Cytological aspects of isolated microspore culture of *Brassica napus. Can. J. Bot.* **66**:1658-1664.

Lesniewska, A., Ponitka, A., Slusarkiewicz-Jarzina, A., Zwierzykowska, Z., Zwierzykowski, A.R., James, H., Thomas, M., and Humphreys, W. (2001). Androgenesis from *Festuca pratensis* x *Lolium multiflorum* amphidiploid cultivars in order to select and stabilize rare gene combinations for grass breeding. *Heredity.* **86**(2): 167-176.

Lichter, R. (1982). Induction of haploid plants from isolated pollen of *Brassica napus. Z. Pflanzenphysiol.* **103**: 229-237.

Lichter, R. (1989). Efficient yield of embryos by culture of isolated microspores of different *Brassicaceae* species. *Plant Breeding.* **103**:119-123

Lionneton, E., W. Beuret, C. Delaitre, S. Ochatt and M. Rancillac (2001). Improved microspore culture and doubled–haploid plant regeneration in the brown condiment mustard (*Brassica j*unce*a). Plant Cell Rep.* **20**: 126-130.

Little, E. L., Megbanua, Z. V., and Parrott, W. A. (2000). A protocol for repetitive somatic embryogenesis from mature peanut epicotyls. *Plant Cell Rep.* **19**: 351-357.

Ma, J. H., Yao, J. L., Cohen, D., and Morris, B. (1998). Ethylene inhibitors enhance *in vitro* root formation from apple shoot cultures. *Plant Cell Rep.* **17**: 211-214.

Maheshwari, S. C., Rashid, A., and Tyagi, A. K.(1982). Haploid from the pollen grains-Retrospect and Prospect. *Am. J. Bot.* **69**:865-879.

Malik, M. R., Rangaswamy, N. S., and Shivanna, K. R. (2001). Induction of microspore embryos in a CMS line of *Brassica juncea* and formation of the androgenic plantlets. *Euphytica.* **120**: 195-203.

Martinez, C., Victoria, F. C., Amezquita, M. C., Tulande, E., Lema, G., and Zeigler R. S. (1996). Comparison of rice lines derived through anther culture and the pedigree method in relation to blast (*Pyricularia grisea Sacc.*) resistance. *Theor. Appl. Genet.* **92** (5): 583-590.

Matsubayashi, M., and Kuranuki, K. (1975). Embryogenic response of the pollen to varied sucrose concentrations in anther culture. *Sci. Rep. Fac. Agric. Kobe. Uni.* **11**: 215-230.

Muranty, H., Sourdille, P., Bernard, S., and Bernard, M. (2002). Genetic characterisation of spontaneous diploid androgenetic wheat and triticale plants. *Plant Breeding.* **121**(6): 470-474.

O'Donoughue, L. S., and Bennett, M. D. (1994). Durum wheat haploid production using maize wide-crossing. *Theor. Appl. Genet.* **89**: 559-556.

Ockendon, D. J. (1984). Anther culture in Brussels sprouts (*Brassica oleracea* var. *gemmifera*). Embryo yield and plant regeneration. *Ann. Appl. Biol.* **105**: 285-295.

Ockendon, D. J. (1988). The ploidy of plants obtained from anther culture of cauliflowers *(Brassica oleracea* var. *botrytis*). *Ann. Appl. Biol.* **113**: 319-325.

Ockendon, D. J. (1988). The ploidy of plants obtained from anther culture of cauliflowers *(Brassica oleracea* var. *botrytis*). *Ann. Appl. Biol.* **113**: 319-325.

Ockendon, D. J.,and McClenaghan, R. (1993). Effect of silver nitrate and 2,4-D on anther culture of Brussels sprouts (*Brassica oleracea* var *gemmifera). Plant Cell Tissue Organ Culture.* **32**: 41-46.

Ouedraogo, J .T., Pierre, C. A. S., Collin, J., Rioux, S., and Comeau, A. (1998). Effect of amino acids, growth regulators and genotype on androgenesis in barley. *Plant Cell Tissue and Organ Culture.* **53**(1): 59-66.

Pauk, J., Kertesz, Z., Beke, B., Bona, L., Csosz, M., Matuz, J. (1995). New winter wheat variety. GK Dekabab developed *via* combining conventional breeding and *in vitro* androgenesis. *Cereal Res. Comm.* **23**: 251-256.

Pechan, P. M., and Smykal, P. (2001). Androgenesis; affecting the fate of the male gametophyte. *Physiologia Plantarum.* **111**(1): 1-8.

Phillips, D. (1995). Cauliflowers. In M. Shoham, Horticulture Australia a complete reference of the Australian horticulture 638A, Morescope publishing Pty. Ltd, Burwood.

Phippen, C., and Ockendon, D. J. (1990). Genotype, plant, bud size, and media factors affecting anther culture of cauliflowers (*Brassica oleracea* var. *botrytis*). *Theor. Appl. Genet.* **79**: 33-38.

Pullman, G. S., Namjoshi, K., and Zhang, Y. (2003). Somatic embryogenesis in loblolly pine (Pinus taeda L.): improving culture initiation with abscisic acid and silver nitrate. *Plant Cell Rep.* **22**: 85-95.

Rae, Howell and Kearsey. (1999). More QTL for flowering time revealed by substitution lines in *Brassica oleracea. Heredity* **83**(5): 586-596.

Ripley, V. L., and Beversdorf, W. D. (2003). Development of self-incompatible *Brassica napus*: (111) *B. napus* genotype effects on S-allele expression. *Plant Breeding.* **122** (1): 1-12.

Romeijn, G., and Lammeren, A. A. M. (1999). Plant regeneration through callus initiation from anthers and ovules of *Scabiosa columbaria*. Plant Cell Tissue Organ Culture. 56(3): 169-177.

Rudolf, K., Bohanec, B., and Hansen. M. (1999). Microspore culture of white cabbage, *Brassica oleracea* var. *capitata* L.; Genetic improvement of non-responding cultivars and effect of genome doubling agents. *Plant Breeding.* **118** (3): 237-241.

Saji, K. V., and Sujatha, M. (1998). Embryogenesis and plant regeneration in anther culture of sunflower (*Helianthus annuus* L.). *Euphytica.* **103**:1-7.

Santos, E. K., Mariath, J.E., Mundstock, E., Hu, C. Y., Zanettini, M. H .B. (1997). Cytological Analysis of early Microspore divisions and embryo formation in cultured soybean anthers. *Plant Cell Tissue Organ Culture.* **49**: 107-115.

Smykal, P., and Pechan, P. M. (2000). Stress, as assessed by the appearance of sHsp transcripts, is required but not sufficient to initiate androgenesis. *Physiologia Plantarum.***110**(1): 135-143.

Stipic, M., and Campion, B. (1997). An improved protocol for androgenesis in cauliflowers (*Brassica oleracea* var. *botrytis*). *Plant Breeding.* **116**:153-157.

Sunderland, N., Collins, G. B., and Dunwell, J. M. (1974). The role of nuclear fusion in pollen embryogenesis of *Datura innoxia. Planta.* **117**: 227-241.

Thurling, N., and Chay, P. M. (1984). The influence of donor plant genotype and environment on production of multicellular microspores in cultured anthers of *Brassica napus* ssp. *oleifera. Ann. Bot.* (London). **54**: 681-693.

Tuvesson S., Ljungberg, A., Johansson, N., Karlsson, K. E., Suijs, L.W. and Josset, J. P. (2000). Large-scale production of Wheat and triticale doubled haploids through the use of a single anther culture method. *Plant Breeding.* **119**(6): 455-4594.

Uzunova, M. I., and Ecke, W. (1999). Abundance, polymorphism and genetic mapping of microsatellites in oil seed rape (*Brassica napus* L.). *Plant Breeding.* **118**(4): 323-326.

Wang, M., Farnham, M. W., and Nannes, J. S. P. (1999). Ploidy of broccoli regenerated from microspore culture versus anther culture. *Plant Breeding.* **118(**3): 249-252.

Williams, J., Pink, D. A. C., and Biddington, N. L. (1990). Effect of silver nitrate on long term culture and regeneration of callus from *Brassica oleracea* var.*gemmifera*. *Plant Cell Tissue and Organ Culture.* **21**: 61-66.

Yang, Q., Chauvin, J. E., and Herve, Y. (1992). A study of factors affecting anther culture of cauliflower (*Brassica Oleracea* var. *botrytis*). *Plant Cell Org. Cult.* **28**: 289-296.

Zaki, M., and Dickinson, H. (1995). Modification of cell development *in vitro*; The effect of colchicines on anther and isolated microspore culture in *Brassica napus*. *Plant Cell Tissue Organ Culture.* **40**: 255-270.

Zamani, G., Kovacs, E., Gouli-Vavdinoudi, D. G., Roupakias, and Barnnabas, B. (2000). Regeneration of fertile doubled haploid plants from colchicines supplemented media in wheat anther culture. *Plant Breeding.* **119**(6): 461- 465.

Zamani, G., Kovacs, E., Gouli-Vavdinoudi, D. G., Roupakias, and Barnnabas, B. (2000). Regeneration of fertile doubled haploid plants from colchicines supplemented media in wheat anther culture. *Plant Breeding.* **119**(6): 461- 465.