BREEDING STRATEGIES IN STURT'S DESERT PEA, *Swainsona formosa* (G.Don) J.Thompson, USING *IN VITRO* AND *IN VIVO* TECHNIQUES

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Sturt's desert pea (*Swainsona formosa*)

DECLARATION

I certify that the substance of this thesis has not already been submitted for any degree and is not being currently submitted for any other degree at this or any other higher education institution.

I certify that to the best of my knowledge any assistance received in preparing this thesis, and all sources used, have been acknowledged.

Zulkarnain

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ABSTRACT

One of the impediments to the commercialisation of Sturt's desert pea (*Swainsona formosa*) as a cut flower is the release of large amounts of pollen grains during anther dehiscence. These pollen grains may stain the petals and, so reduce the quality of the flowers. In addition, self-pollination can occur during transportation causing flowers to degenerate quickly. This project was undertaken with the objective to investigate strategies to overcome such problems through plant a breeding approach. Two strategies were employed, the induction of haploid plant production via anther culture and the induction of tetraploid plants using colchicine and oryzalin. Crossing diploid with tetraploid plants leads to the production of triploid plants. Either haploids or triploids are sterile and produce no pollen grains. The results of these two strategies are presented in this thesis.

The determination of chromosome number is a prerequisite step for both strategies. This study showed that by using root tip and anther squashes, the number of chromosomes in *S. formosa* was $2n = 2x = 16$. This finding confirmed the base chromosome number of $x = 8$ in the tribe Galegeae.

Understanding plant embryology is not only crucial for taxonomic purposes but also for breeding programmes. Therefore, the embryological development and sexual reproduction of *S. formosa* was also investigated. The anthers of *S. formosa* were found to be tetrasporangiate, with a 3-layered wall below the epidermis. The wall was comprised of a layer of endothecium, middle layer and secretory tapetum. Pollen grains were triporate and shed at a 2-cell stage. The ovules were campylotropous with a zigzag micropyle. Multiple embryo sacs were occasionally found but only one mature embryo was formed in the seed. The seed coat was composed of a single layer of thick-walled palisade cells on the outside followed by a single layer hypodermal sclereids on the inside. Seed germination was epigeal.

In order to support the breeding programme of *S. formosa,* methods of pollen preservation were also developed. It was found that pollen grains could be stored at 4°C for up to 28 days without significantly losing their viability. Pollen longevity could be extended beyond 2 months when stored at -10°C and under dry conditions. These findings provided a simple and economically sound method for storage of *S. formosa* pollen. Although pollination was often prevented by the presence of stigmatic cuticle, the stigma was found to be receptive from one day before anther dehiscence. The receptivity reached its peak within 4 days after anther dehiscence. These results provide a valuable background to the conventional breeding of this species to create hybrids through cross-pollination.

The size of floral buds has been effectively used as a morphological predictor to determine the stage of microspore development within anthers in *S. formosa*. Therefore, anther preparation during subsequent experiments relied on this indicator. Of the variables tested, auxin and cytokinin were found to play a key role in anther development following culture initiation. Callus proliferation was enhanced by pre-treating the anthers with mannitol starvation at 4°C followed

by introduction to a double-phase medium supplemented with Ficoll-400™. Embryogenic callus was produced and embryogenesis was detected but these embryos did not originate from microspores. Instead, the embryos grew from sporophytic tissue of the anther wall. The embryos failed to develop further when subcultured to root and shoot induction medium due to a high frequency of hyperhydration. Morphogenesis was detected but shoots and roots developed poorly. This investigation also revealed that proline could be used as a biochemical indicator for early detection of embryogenesis.

Since the induction of polyploidysation will only be effective if the treatment is given during the active vegetative stage, the conversion from vegetative to reproductive growth in *S. formosa* was investigated with particular emphasis on the effect of different photoperiods. Although photoperiod affected the time required for first flower initiation, the number of nodes before the plant entering the reproductive stage was not affected by photoperiod and presumed to be set genetically. It was found that under artificial photoperiods of 16, 12 and 8 hours the 12th and 11th nodes were the critical nodes for the main and side stems, respectively. Meanwhile the 10th and 8th nodes were found to be critical for main and side stems of plants grown under natural photoperiods ranging from 12 to 16 hours during their life cycle. Histological examination indicated that when the plants were grown under 12 – 16 hours photoperiods the first floral bud formation was initiated within 56 – 60 days after germination, thus this period was considered as the critical time for the conversion from vegetative to reproductive growth in *S. formosa.*

Following from the previous experiments, polyploidy induction using antimitotic chemicals was carried out as early as possible in the life cycle of the plant. However, seed treatment with colchicine was found to be detrimental and resulted in no polyploid induction. Seedlings, in contrast, were found to be more responsive to either colchicine or oryzalin application. Unfortunately, the rate of conversion was very low. It was also noted that the ployploidysation in *S. formosa* was unstable. Reversion to diploid characteristics in F1 generation has become a major problem in obtaining plants with stable tetraploid chromosomes.

Some new findings relating to the breeding aspects of *S. formosa* are reported here although the induction of haploid and triploid plants was unsuccessful. These results can, however, be used as a valuable reference for future work on breeding programmes to create quality flowers in *S. formosa.*

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CHAPTER 1
GENERAL INTRODUCTION

Swainsona formosa (G.Don) J.Thompson (Sturt's desert pea), known to aborigines as Marlukuru (Bindon, 1996), is one of Australia's most spectacular wild flowers, and is the floral emblem of South Australia (Kirby, 1996a). Its large flag-shaped flowers, coloured bright red (or pure white to deep purple in some wild populations), have made this plant one of most striking flowering plants in the world (Taji, 1997).

The economic importance of *S. formosa* is in its potential use in a hanging basket or container plant, or as a cut flower plant (Williams and Taji, 1991; Kirby, 1996a, 1996b). It is not only in demand in the domestic cut flower market, but also overseas. A market survey conducted by South Australian Agriculture revealed that Japan is one of the most important cut flower markets in the world to which *S. formosa* has already been exported (Barth and Bennel, 1989). Since the market interest is in lighter coloured flowers rather than in the usual brilliant red and black flowers (Taji, 1997), research conducted by Taji since 1997 has focussed on *S. formosa* breeding to create lines with these light coloured flowers that are keenly sought by markets in many Asian countries including Japan.

Impediments to the commercialisation of *S. formosa* as a cut flower include the production of large amounts of pollen grains and delicate petals that are easily bruised (Barth, 1990). Flower quality is reduced due to petal staining by pollen grains released from the anthers during transportation. In addition, Kirby (1996b) claimed that self-pollination of the flowers during transportation occurs easily. Degeneration of flowers usually occurs following pollination.

Thus, like other cut flowers, commercialisation of *S. formosa* depends upon the ability of a breeding programme to obtain cultivars that meet market interests. This study was conducted to investigate the limitations in the breeding programme of *S. formosa*.

1.1 Thesis outline

An overview of the work is given in Chapter 1 (General introduction). It starts with a description of the general features of *S. formosa*, followed by discussion of its economic importance and problems associated with the commercialisation of this plant. The objectives of the whole project are also described in this chapter.

A critical review of the significance of male-sterility in ornamental plants, followed by factors affecting the occurrence of male-sterility is presented in Chapter 2 (Literature review). The potential of ploidysation for crop improvement is reviewed and the methods used are considered, i.e. anther culture and the application of colchicine and oryzalin in polyploidy induction. Embryology and the determination of timing of conversion from vegetative buds to floral buds in *S. formosa* are also reviewed as these two aspects are closely related to the topic of the project.

A brief description of *S. formosa* is given in Chapter 3 (The *Swainsona formosa* plant). The description is generally restricted to important agronomic characteristics and floral development.

Materials and methods used in the overall work are described in general in Chapter 4 (General materials and methods). Further information is given on each experiment in subsequent chapters.

The investigation on chromosome number in *S. formosa* is described in Chapter 5 (Chromosome number). This work reveals the chromosome number in plant materials used throughout the project. Information from this trial is important for evaluating the effectiveness of treatments in the production of haploid, triploid or tetraploid plants.

Embryology is another fundamental aspect in understanding plant breeding. Therefore, the embryological development in the sexual reproduction of *S. formosa* was studied and the results are presented in Chapter 6 (Embryological development and sexual reproduction).

The work of determining the duration of pollen viability and the duration of stigma receptivity, either in glasshouse conditions or cold storage is presented in

Chapter 7 (Pollen longevity and stigma receptivity). This work is important as an initial step in conventional breeding and to ensure the availability of viable pollen grains when the stigmas are receptive.

The use of anther culture in the production of haploid plants is described in Chapter 8 (Anther culture). A number of treatments such as developmental stage of microspores, media types, light spectra, anther pre-treatment, plant growth regulators and the addition of Ficoll have been tested and results are discussed.

Understanding the correct timing of conversion from vegetative to floral buds would be of great importance to the successful modification of subsequent plant growth and development. In the context of this thesis, learning about the conversion time from vegetative to floral buds may be useful in determining the correct timing for the application of antimitotic chemicals used in the manipulation of ploidy level in plants. Chapter 9 (Conversion from vegetative buds to floral buds) deals with experiments in growth chambers as well as microtechnique work exploring the exact time of the conversion of vegetative buds to floral buds in *S. formosa.* This work incorporated three different photoperiods.

The induction of tetraploid plants was attempted by using antimitotic chemicals. Various concentrations of colchicine and oryzalin application have been tested. The results are presented and discussed in Chapter 10 (Polyploidy induction).

The results of the experiment are summarised, and possible explanations for the strategies of producing male-sterile plants are discussed in Chapter 11 (Integration and future directions). Practical recommendations arising from the results of these studies and suggestions for further research are made.

1.2 The objectives of the project

This study was aimed at understanding the aspects related to the production of male-sterile flowers in *S. formosa* using *in vivo* and *in vitro* strategies. The *in vitro* strategy is centred on the production of haploid plants using anther culture. Since pollen grains are haploid, plants regenerated from them are also haploid.

The objective of the *in vivo* strategy is to produce triploid plants. The first step in the process is to produce plants that are tetraploid, by the application of an antimitotic chemicals such as colchicine or oryzalin that act by disrupting spindle formation during mitosis. The flowers of treated plants, upon pollination and fertilisation, produce seeds that are tetraploid. Crossing diploid, which is the common ploidy in *S. formosa*, and tetraploid individuals results in the production of triploid plants.

This research has lead to publication of several refereed papers in two proceedings of two international conferences and in an international journal. A list of these publications is in Appendix 1.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Swainsona formosa is one of Australia's native plants, and is one of the world's most spectacular flowering plants. The outstanding feature of *S. formosa* is its brilliant-coloured flowers, from white or pink through to dark red standard and keel with or without a distinctive boss.

The great potential of *S. formosa* is as a container-grown ornamental, either in a flowering pot or hanging basket, or as a cut flower. With ideal growing conditions and intensive plant care, *S. formosa* can also be spectacular used as a garden ornamental (Williams, 1996). The commercialisation of *S. formosa* for the cut flower market is quite flexible. It can be marketed either as individual, single-stemmed flower clusters or as multiple clusters on a leafy branch. Asian markets, particularly Tokyo and Osaka in Japan, are a potential destination for trade in *S. formosa* (Grootenboer, 2000).

However, the commercialisation of *S. formosa* as a cut flower is subject to a number of limitations. In addition to colour variations, stem length and the number of blooms per cluster, another impediment to commercialisation of *S. formosa* as a cut flower is the production of large amounts of pollen in the flowers. This brings about a reduction in flower quality due to petal staining by the pollen grains that are shed during transportation. In addition, during transportation pollination may occur, resulting in the rapid degeneration of pollinated flowers and thus reduction in the vase-life of the flowers. Flowers of *S. formosa* start to senesce on the plant within 2 - 3 days after effective pollination. Unpollinated flowers, on the other hand, will remain fresh on the plant for 5 – 10 days (Williams, 1996). This indicates that avoiding pollination will increase the vase-life of harvested flowers.

Research on the breeding of *S. formosa* has been conducted with the emphasis on the production of male-sterile flowers. Two strategies have been used, an *in vitro* technique aimed at producing haploid plants and an *in vivo*

technique aimed at producing triploid plants. Both haploid and triploid plants are sterile because where there is an odd number of chromosome sets reproductive fertility is usually impaired. This is because during meiosis the normal pairing of chromosomes cannot properly take place since one set of chromosomes has no homologous set with which to pair, and so gametes fail to form.

This review begins with the discussion on the significance of male-sterility, particularly in ornamental plants. A number of factors affecting male-sterility in plants are then reviewed. The potential of ploidy modification for crop improvement is described and the methods used in modifying ploidy level, including haploidysation via anther culture and chromosomes doubling by the use of chemicals, are discussed. Plant embryology as a fundamental aspect in plant breeding is also reviewed and discussed with particular emphasis in legumes.

2.2 The significance of male-sterility in crop plants

The term male-sterility is used to indicate plant sterility due to the failure of male gametophyte to function. Haploid plants refer to individuals having half the normal number of chromosomes in their somatic ($2n = x$) tissues. Triploid plants are those having chromosome numbers in their somatic tissues of $2n = 3x$. Both haploid and triploid plants are sterile because where there is an odd number in the chromosome set, reproductive fertility is usually impaired. This is because during meiosis the normal pairing of chromosome cannot properly take place since one set of chromosomes will have no homologous set to pair with, and as such gametes fail to form.

The normal development of the male reproductive organ (stamen) and the male gametophyte (pollen grain) is essential for the successful completion of sexual reproduction in angiosperms. However, in some plants pollen may be aborted, stamens malformed, or stamens may be lacking entirely (Lersten, 1980) due to chromosomal aberrations, gene action, or cytoplasmic influences (Allard, 1960), resulting in male-sterility.

Sawhney (1997) stated that male-sterility is a wide occurrence in flowering plants. Although this system has a genetic disadvantage since it only contributes

genes to the next generation via ovules (Poot, 1997), it has been of interest to various plant biologists and plant breeders. Male-sterile mutants serve as useful tools for investigations into the genetic, molecular, physiological, and developmental processes involved in stamen and pollen development. For plant breeders, male-sterile plants are useful systems for interspecific hybridisation and for performing backcrosses. The use of male-sterile plants eliminates the labour-intensive process of flower emasculation, and therefore significantly reduces the cost of hybrid seed production. A high rate of homozigosity exists in the hybrid seeds produced using doubled-haploid plants as a result of anther culture (Bayliss *et al.*, 2002).

A number of abnormalities are frequently found in a male-sterile system. Singh (1993) reported that depending on the source of the sterility, male-sterile plants generally have smaller, shrunken and non-dehiscent anthers. In pigeon pea, Saxena and Singh (1996) found different characteristics of male-sterility caused by different sources. When male-sterility was caused by non-separation of tetrads associated with a persistent tapetum and intercellular walls of the two adjacent microsporangia, male-sterile populations were characterised by translucent anthers. The degeneration of the tapetum by the vacuolation during the first division of meiosis results in shrivelled, arrowhead-shaped, non-dehiscent, brown anthers. Partial male-sterility was also found in pigeon pea due to a partial collapse of fully developed tetrads, and the plants were characterised by limited amounts of pollen grains within each flower. In *Brassica oleracea*, the pollen formation ceases at tetrad stage, and at the time of anthesis the anthers shrivel, curve, and are smaller in size and pale in colour (Rai, 1996).

Allard (1960) and Lawrence (1968) classified male-sterility into three categories based on the way it is genetically controlled, i.e.: genetic, cytoplasmic and genetic-cytoplasmic. Genetic male-sterility is a common phenomenon in flowering plants and recorded in most of the major crop plants such as *Glycine max* and *Hordeum vulgare* (Stuber, 1980), *Brassica napus* (Shukla and Sawhney, 1994; Singh and Sawhney, 1998), *Cajanus cajun* (Saxena and Singh, 1996) and *Lycopersicum esculentum* (Singh and Sawhney, 1998). Genetic male-sterile

plants produce non-functional stamens or have no pollen grains and this may be due to several factors. One of these factors is termed structural, and includes malformation or absence of stamens and non-dehiscence of anthers. The second type is termed functional. Plants with this type of male-sterility are capable of producing viable pollen but barriers such as faulty exine or lack of exine formation prevent anther dehiscence. The third basis of genetic male-sterility is termed sporogenous, and occurs in plants as a result of an extreme scarcity of pollen due to the abortion of microsporogenous cells during meiotic processes (Singh, 1993). Genetic male-sterility can be used to develop improved combinations of genes in some species. It has been successfully used in obtaining natural crosses in crops such as barley and soybean (Stuber, 1980).

Cytoplasmic male-sterility, like genetic male-sterility, also occurs in a wide range of crop plants and is maternally inherited (Singh, 1993; McVetty, 1996). Cytoplasmic male-sterile plants are capable of producing seeds when pollinated. However, these seeds will only produce male-sterile offspring since their cytoplasm is derived entirely from the female gamete (Allard, 1960). It has been recorded in plants such as *L. esculentum* (Singh and Sawhney, 1998), *B. napus* (Bellaoui *et al.*, 1997; Singh and Sawhney, 1998), *Phaseolus vulgaris* (Janska *et al.*, 1998; Sarria *et al.*, 1998) and *Helianthus annuus* (Gagliardi and Leaver, 1999). This type of male-sterility is the most convenient and is being used extensively in hybrid seed production for many crops such as *Zea mays*, *Sorghum bicolor*, *Beta vulgaris*, *Pennisetum glaucum* and *H. annuus* (Forsberg and Smith, 1980). It has real advantages in certain ornamental plants due to the fact that the offspring are also male-sterile, bloom longer and the flowers remain fresh longer too. This would obviously be an advantageous feature in flowering ornamental plants such as *S. formosa*.

The third type, cytoplasmic-genetic male-sterility, involves the interaction of genetic (nuclear) and cytoplasmic factors (Singh, 1993). Genetic-cytoplasmic male-sterility has been used extensively in commercial hybrid seed production (Stuber, 1980).

2.3 The potential of ploidy manipulation in crop improvement

The rule that somatic cells are 2*x* and gametes are *x* is subjected to a number of exceptions that have important effects on plant breeding. Allard (1960) classified these exceptional cases into two major groups. In the first, the organism is characterised by an unusual number of repetitions of one or a few particular chromosomes in the compliment. In the second, the deviation is in the form of an unusual number of repetitions of the full complement of chromosomes. The main features of the terminology to describe these chromosomal repetitions, the effect of ploidysation and its importance in crop plants are reviewed.

2.3.1 Terminologies

The term *ploidy* is used to describe the number of chromosome sets in the nuclei of a cell. Therefore, the term *ploidysation* refers to the process of the induction of modification of the status of the chromosome sets in that organism. Two widely used terms of ploidysation in plant breeding include *haploidysation* i.e. the induction of one set of chromosomes, and *polyploidysation* i.e. the induction of three or more sets of chromosomes.

The term *aneuploidy* describes organisms whose chromosome number is not a whole number multiple of the basic number of the group. For example, if the basic number of chromosome of a species is *n* and the normal complement is 2*x,* a deviate having 2*x* plus or minus one or more chromosomes is then called an *aneuploid.* There are possible combinations of *aneuploid* such as *nullisomic, trisomic* and *tetrasomic* and their modifications. *Nullisomic* refers to the loss of both members of one pair of a normal chromosome complement. If one particular chromosome is present in triplicate, that is two complete sets of chromosomes plus a single extra chromosome, the organism is called *trisomic.* The term *tetrasomic* is applied to organisms having two extra chromosomes of one particular chromosome (Allard, 1960).

Euploid is the term describing the number of repetitions of entire chromosome sets. An organism is called *haploid* if it has only a single

complement of the basic chromosome set of the species. A *diploid* has two full sets, a *triploid* has three, a *tetraploid* has four, a *pentaploid* has five, and so on. Based on the degree of differentiation of repeated chromosome sets, there are further distinctions among *euploids.* If each of repeated chromosome sets is identical or closely similar to each other, the organisms are called *autopolyploids.* On the other hand, in *allopolyploids* the two or more basic sets of chromosomes making up the multiple set of chromosomes are differentiated from each other (Allard, 1960). Table 1 illustrates the complete basic terminologies of ploidysation.

Table 1.
Guide to the terminology of ploidysation (A, B, C are non-homologous chromosomes) (Allard, 1960).

Term	Formula	Somatic chromosome complement
Aneuploids:		
nullisomic	$2x - 2$	(AB) (AB)
monosomic	$2x - 1$	(ABC) (AB)
double monosomic	$2x - 1 - 1$	(AB) (AC)
trisomic	$2x + 1$	(ABC) (ABC) (C)
double trisomic	$2x + 1 + 1$	(ABC) (ABC) (A) (B)
tetrasomic	$2x + 2$	(ABC) (ABC) (A) (A)
monosomic-trisomic	$2x - 1 + 1$	(ABC) (AB) (A)
Euploids:		
haploid	x	(ABC)
diploid	$2x$	(ABC) (ABC)
triploid	$3x$	(ABC) (ABC) (ABC)
autotetraploid	$4x$	(ABC) (ABC) (ABC) (ABC)
allotetraploid	$2x + 2x'$	(ABC) (ABC) (A'B'C') (A'B'C') or (ABC) (ABC) (DEF) (DEF)

The somatic chromosome number of any form here is designated $2n$ and its gametic chromosome number is n, unless it is an aneuploid.

2.3.2 The effect of ploidy changes on plant morphology and productivity

Chase (1974) claimed that the primary advantage of plant breeding using haploidysation is the possibility of isolating an array of individual genomes for selection, study, multiplication and recombination. In addition, the possibility of

obtaining pure line cultivars also takes less time than conventional methods. Ploidy manipulation through the production of haploid plants by anther or microspore culture is an effective method to obtain doubled-haploid clones with good tuber yield and tuber appearance in potato (Wang and Ran, 2000). Doubled-haploid technology has also been effective in improving agronomic performances in *Triticum aestivum* (Broughton *et al.*, 2002) and *H. vulgare* (Kasha *et al.*, 2001; Davies *et al.*, 2002).

Triploid plants, which are usually sterile due to the absence of pollen grains can be quite interesting in breeding programmes. There are a few crops in which triploidy may be valuable. *Humulus lupulus*, *Citrus sinensis* (Simmonds and Smart, 1979) and *Citrulus vulgaris* (McCuistion and Wehner, 2002) are examples of plant species where triploidy is beneficial. In cut flower crops such as *S. formosa* triploidy would give benefits because the absence of pollen grains makes the flowers bloom longer and the problem of petal staining can be avoided. Triploids can be obtained *in vitro* by the induction of shoot regeneration directly from endosperm (Pierik, 1997), whereas *in vivo* triploids can be produced by crossing a diploid with a tetraploid individual (McCuistion and Wehner, 2002).

A tetraploid can be formed with tissue culture by adventitious shoot formation, or by treating a diploid individual with antimitotic chemicals. Pierik (1997) proposed two possible explanations for the development of tetraploids via adventitious shoot formation. Firstly, there are naturally single tetraploid cells present in a diploid individual. Since adventitious shoot formation usually occurs from a single cell layer, complete tetraploids can be obtained. Secondly, adventitious shoot formation does not involve chromosome separation after cell division, possibly due to the presence of antimitotic chemical such as colchicine or oryzalin, resulting in tetraploid individuals.

The production of tetraploid plants with the use of antimitotic chemicals such as colchicine has been successful in a wide range of plant species (Saxena and Singh, 1996; Smith and Hamill, 1996; Takamura and Miyajima, 1996; Azhar *et al.*, 2002). Colchicine inhibits spindle formation and prevents chromosome

separation at anaphase, resulting in a daughter cell with double the chromosome number (see section 2.5 for more on effects of colchicine).

In comparison with diploids or triploids, tetraploids are often larger and lusher in growth, and because of multivalent formation at meiosis, always initially less seed-fertile. This suggests that tetraploidy would give a greater benefit for plants grown for vegetative products (Simmonds and Smart, 1979), such as forage species and a number of vegetable crops such as cabbage and spinach. However, Saxena and Singh (1996) reported that colchicine-induced tetraploid pigeon pea showed poor growth and less vigour compared to diploids. In addition, the tetraploids were shorter, with fewer nodes, thicker leaves and stems, and lower shoot dry weight per plant, than those of diploids. The tetraploids also had thicker leaves, larger but fewer (per unit area) stomata, larger flowers and floral parts, larger pollen grain with relatively higher pollen sterility, and poor pod set. With ginger, the tetraploids had fewer but longer stems and leaves, and were similar in many respects to the diploids propagated conventionally using rhizome sections. The tetraploids in ginger also produced longer rhizomes with a greater knob size (Smith and Hamill, 1996). For floral crops, the induction of artificial tetraploids represents a useful tool since larger flowers, longer flowering periods and deeper colours can be obtained through chromosome doubling (Väinölä, 2000). The following table gives chromosome numbers for some legume species.

Table 2.
Somatic (2*n*) chromosome numbers of some legume species.

Species	Chromosome number	Reference
Acacia constricta Gray.	26	Ward (1984)
Arachis hypogaea	20	Paterson *et al.* (2000)
Carmichaelia nana Colenso ex Hook.f.	32	Dawson and Beuzenberg (2000)
Cassia roemeriana Scheele.	28	Ward (1984)
Cicer arietinum L.	16	Ward (1984)
Clianthus puniceus var. *maximus* (Colenso) Kirk.	32	Dawson and Beuzenberg (2000)
C. puniceus 'Albus'	32	Dawson and Beuzenberg (2000)
Corallospartium crassicaule var. *racemosum* Kirk.	32	Dawson (1995)
Crotalaria bupleurifolia Schlechtendal & Chamisso.	32	Windler (1974)

Species	Chromosome number	Reference
Dalea jamesii (Torr.) Torr. & Gray.	14	Ward (1984)
Glycine max L. (Merr).	20	Paterson *et al.* (2000)
Lotus humistratus Greene.	14	Ward (1984)
Lupinus laetus W. & S.	48	Ward (1984)
Medicago sativa L.	32	Paterson *et al.* (2000)
Pisum sativum L.	14	Paterson *et al.* (2000)
Phaseolus vulgaris L.	22	Paterson *et al.* (2000)
Rhynchosia texana Torr. & Gray.	22	Ward (1984)
Swainsona galegifolia (Andr.) R.Br.	32	Ermayanti *et al.* (1993)
S. novae-zelandiae Hook.f.	32	Heenan (1998a)
Trifolium andinum Nutt.	16	Mosquin and Gillet (1965)
T. latifolium (Hook.) Greene.	16, 32	Mosquin and Gillet (1965)
T. longipes Nutt.	16, 32, 48	Mosquin and Gillet (1965)
T. parryi Gray subsp. *salictorum* (Greene ex Rydb.) J. M. Gillet.	32	Mosquin and Gillet (1965)
Vicia exigua Nutt. ex Torr. & Gray.	22	Ward (1984)
Vigna radiata (L.) Wilczek.	22	Paterson *et al.* (2000)

2.4 Anther culture

Obtaining homozygous lines from highly outcrossing species such as *S. formosa* would take a long time (Williams and Taji, 1992). The process starts with a cross-pollination to combine desirable parental traits resulting in heterozygous but genetically uniform offspring. The reproduction of these offspring is frequently accompanied by the separation of homologous chromosomes and genes from different parents at meiosis, and produces genetic variability within the population of the next generation (Croughan, 1995). Therefore, the overall process of producing homozygous lines as predicted by Ferrie and Keller (1995) may take 10 years or even more, depending on the plant species.

In contrast, the use of microspore embryogenesis via anther or microspore culture is valuable for the detection of recessive gene traits and the exploitation of gametoclonal plants (Christou, 1992). More importantly, this technique offers the opportunity to generate purely homozygous lines more rapidly and efficiently than with conventional ways (Tomasi *et al.*, 1999). Immature pollen or microspores contained within the anther may give rise to embryos or to callus tissue, which then is induced to regenerate complete plants under favourable conditions. Since pollen is haploid, plants regenerated from pollen cells will also be haploid.

Haploid plants are sterile and can produce no seeds. Through chromosome doubling treatment it is possible to produce homozygous, fertile, doubled-haploid and pure breeding lines (Ferrie and Keller, 1995). Thus, microspore embryogenesis makes mutational breeding and selection of beneficial traits possible (Tomasi *et al.*, 1999).

Ovules are also a possible alternative source for haploid or doubled-haploid production (Sato *et al.*, 2000). However, the exploration of this tissue in producing haploid or doubled-haploids in breeding programmes is still very limited.

2.4.1 Mechanism of plant regeneration via androgenesis

The term androgenesis refers to plant regeneration directly from microspore culture under *in vitro* conditions. The underlying principle of androgenesis is to stop the development of the pollen cells, which normally become sexual cells, and to force their development directly into a complete plant (Nitsch, 1981). This process inhibits typical gametophytic differentiation and instead allows cell division and regeneration to occur (Dunwell, 1986).

As gametogenesis (microspore development) takes place, mature pollen grains are formed via mitosis. Since the developmental route is not yet determined during microgametogenesis, there is a chance to interrupt the normal gametophytic pathway and to induce sporophytic development. Vicente *et al.* (1992) and Mitykó *et al.* (1996) suggested that microspores within the range of the uninucleate to mid-binucleate stage were suitable materials for the induction of haploid sporophytic development in a wide range of plant species. As the result of sporophytic divisions, multicellular microspores develop within the anthers. Differentiation of these multicellular units may result in pollen embryos, which then develop into haploid plants.

2.4.2 Factors affecting the induction of microspore embryo

Anther culture has considerable potential in plant breeding due to its potential to produce haploid plants from immature microspores, which can be

doubled to obtain fertile and true-to-type doubled-haploid individuals. Anther culture has been successfully applied in plants such as, *Linum usitatissimum* (Nichterlein and Friedt, 1993; Chen *et al.*, 1998a), *Populus trichocarpa* (Baldursson *et al.*, 1993), *Raphanus sativus* (Takahata *et al.*, 1996), *Capsicum annuum* (Mitykó *et al.*, 1996). However, many others have not; and even among those that do respond there is a considerable variation in embryogenesis (Zhong *et al.*, 1995; Zhao *et al.*, 1996; Saïdi *et al.*, 1997).

For it to be successfully applied in plant breeding, a high yield of plants must be obtained consistently from anther culture. There are a number of factors associated with the success of anther culture (Sunderland, 1974; Dodds and Roberts, 1985). The following sections are aimed at reviewing factors that play significant role in the success of androgenesis.

2.4.2.1 Plant genotype

It is obvious that the response of anthers during *in vitro* culture is very dependent upon the genotype of the donor plants. Palmer and Keller (1997) added that the genotype of donor plant affected not only the frequency of embryogenesis but also the quality of the embryo produced.

The significant genotype dependence in androgenic response is reported by Mitykó *et al.* (1996) on pepper anther culture. They found that large-fruited genotypes produced the highest number of plantlets per cultured anther compared to small- and medium-fruited genotypes, which proved to be poor or non-responsive. Genotypic-dependence is also reported in *R. sativus* (Takahata *et al.*, 1996), *T. aestivum* (Bitsch *et al.*, 1998), *L. usitatissimum* (Chen *et al.*, 1998b), *Scabiosa columbaria* (Romeijn and Lammeren, 1999) and *Phleum pratense* (Guo *et al.*, 1999). Although the basis of genetic control remains unexplained, it is clear that genetic factors interact with other factors to control pollen embryogenesis (Palmer and Keller, 1997).

2.4.2.2 The age of the donor plant

The age of the donor plant is another key factor affecting microspore embryogenesis. This may be attributed to variations in the content of endogenous plant growth regulators as well as biochemical processes within the anther during bud development. However, it seems that the effect of the age of donor plant is species-dependent. In *Aesculus carnae,* Marinkovic and Radojevic (1992) reported that higher frequencies of embryogenesis occurred with microspores from older plants. Meanwhile, in *C. annuum,* Kristiansen and Andersen (1993) found that younger plants yielded more responsive microspores than the older ones. Therefore, different ages affect responsiveness of microspores in different species.

2.4.2.3 Environmental conditions of the donor plant

Although it is impossible to make a general recommendation about optimal growth conditions, it is clear that anther respond differently to environmental factors under which donor plants were grown. Factors such as temperature and photoperiod (Dunwell, 1986) may interact with plant genotype and affect the frequency of pollen embryogenesis.

Kristiansen and Andersen (1993) found that in *C. annuum* the optimal temperature for donor plants were 26°C. For most species, plants grown under low temperature regimes yielded more responsive microspores (Palmer and Keller, 1997). Guo *et al.* (1999) reported that embryo production in *P. pratense* was increased when the donor plants were grown under low temperature. Low temperature pre-treatments of donor plants are probably important in arresting gametophytic development of microspores as cytological modifications have been noted in embryogenic microspores from plants maintained at low temperature.

In *Nicotiana tabacum* cv. White Burley, the most productive anthers came from plants grown at 20°C under a 8-h photoperiod (Dunwell, 1974). However, photoperiod had no significant effect on the embryo formation from anther culture of *C. annuum* (Kristiansen and Andersen, 1993).

2.4.2.4 Developmental stage of the microspore

The particular stage of microspore development at the time of culture initiation plays an important role in achieving success with microspore embryogenesis. Small differences in the developmental stage may produce great differences in the result. Palmer and Keller (1997) suggested that the late-uninucleate to early-binucleate stage appears to be optimum for the induction of embryo formation. However, the exact stage of microspore development, which is more readily diverted to a sporophytic pathway, seems to vary with species. In *N. tabacum,* Sunderland (1974) reported that anther response increased sharply during the microspore phase and reached its peak at the first pollen mitosis. The late-uninucleate stage was found to produce the highest number of plantlets per anther. Microspores at the late-uninucleate stage were also used by Nichterlein and Friedt (1993) in their study on microspore culture of *L. usitatissimum*. However, with *P. pratense,* Guo *et al.* (1999) found that the optimum stage for microspore development was between the late-uninucleate and binucleate stages.

Although many workers proposed the generalisation that the optimum period for pollen response lies in between the tetrad and binucleate stages, an exact determination of microspore stage before being introduced to culture requires a cytological analysis. As such, for practical programmes many workers rely on morphological parameters such as the length of floral buds and petals (Mitykó *et al.*, 1996; Takahata *et al.*, 1996; Tomasi *et al.*, 1999). Though the optimum bud size varies among genotypes, the microspore populations represented in these buds are mostly in the range of uninucleate and binucleate stages.

In conjunction with the best response in culture, Dodds and Roberts (1985) proposed three categories of microspore developmental stages: premitotic, mitotic, and postmitotic. Plants in the premitotic category show best embryoid formation when microspores have completed meiosis but have not yet started the first pollen division (e.g. *Hyoscyamus niger* and *Hordeum vulgare*). Plants belonging to the mitotic group respond best about the time of the first pollen division (eg. *N. tabacum, Datura innoxia* and *Paeonia* spp.). Meanwhile, plants

in the postmitotic category show the best response when microspores are in the early-binucleate stage (eg. *Atropa belladonna*).

2.4.2.5 Culture environment

In addition to the plant material mentioned in the previous sections, the environmental factors under which the cultures are maintained are also critical for the success of microspore embryogenesis. Some key factors for the induction of androgenesis are briefly discussed below.

Light

Light plays an important role in the induction of androgenesis but the response shown by cultured anthers varies with species. Sopory and Maheshwari (1976) reported the promotive effect of light on anther culture of *D. innoxia.* Sopory *et al*. (1978) also reported similar results in *Solanum tuberosum.* In some species the frequency of haploid formation and plantlet growth are better in light conditions than in conditions of darkness (Nitsch, 1977). For example in pepper (Mitykó *et al.*, 1996) a photoperiod of 12-h with approximately 37 µmol m^{-2} s^{-1} light intensity was the most effective for microspore embryogenesis. In anther culture of *N. tabacum,* however, initial incubation in dark conditions is essential for embryoid formation (Sundherland and Roberts, 1977). These differences in light requirements among plant species may be attributed to the changes in endogenous levels of plant hormones, particularly auxin, in response to light.

Temperature

In general, temperature for *in vitro* culture is set between 25 to 30ºC, depending on the type of culture and the purpose of the programme. The initial temperature is an essential factor for pollen embryogenesis though it seems to be species-dependent. In spring wheat (McGregor and McHughen, 1990) temperature treatments showed a significant effect on androgenesis. Maximum callus induction and regeneration were obtained when anthers were cultured for 14 or 21 days at 30ºC before transfer to 28ºC. In anther culture of *C. annuum*,

initial incubation in dark conditions for 8 days at 35ºC was required before the cultures were exposed to a 12-h photoperiod at 25ºC (Mitykó *et al.*, 1996). Similarly, in flax (*L. usitatissimum*), the culture of anthers for one day at 35ºC prior to continuous culture at 25ºC in darkness significantly increased the efficiency of plant regeneration (Chen *et al.*, 1998b). It has been suggested that the elevated temperature inhibits protein synthesis, leading to sporophytic induction. Alternatively, heat-shock may also cause the destruction of the pre-existing post-transcriptional control apparatus in the microspore. Bate *et al.* (1996) suggested that in certain cases the translation of pollen-specific gene transcripts was enhanced developmentally, while the transcript of other genes may be temporally inhibited until later stage (Curie and MacCormick, 1997). The destruction of these post-transcriptional control mechanisms may contribute to the re-programming of the microspore, which normally develops into a sexual cell, to become a complete plant.

Mineral nutrition

The importance of particular mineral nutrition in microspore embryogenesis is obvious as successful embryoid production of different species is achieved on media of different compositions. The common medium employed in most tissue culture systems including anther culture is MS medium (Murashige and Skoog, 1962). However, other media are also used such as WH (White, 1962), LS (Linsmaier and Skoog, 1965), B5 (Gamborg *et al.*, 1968), Nitsch's (Nitsch, 1969), SH (Schenk and Hildebrandt, 1972), WPM (Lloyd and McCown, 1980), R2M (Wang and Hu, 1984) and FHG (Cistué *et al.*, 1995).

The nutritional requirements of isolated microspores are more complex than those of excised anthers. In isolated microspores certain factors responsible for the induction of androgenesis, which might have been provided by the anther, are missing, and these have to be provided through the culture medium (Reinert and Bajaj, 1977). For example, tobacco excised anthers can be successfully cultured on a simple basal medium, whereas the isolated microspores require a higher amount of nitrogen in the form of amino acids (Reinert *et al.*, 1975).

Microelements, particularly iron in the form of Fe-EDTA, are found to be important in microspore embryogenesis. In the work on tobacco microspore culture (Kyo, 1990) and anther culture of *Anemone canadensis* (Johansson *et al.*, 1990) the addition of Fe-EDTA was found to enhance microspore embryogenesis. Ethylenediamine tetraacetic acid (EDTA) is a chelating compound, in which iron is bound but it is still available at pH up to 8.0 during culture growth. The availability of Fe may rapidly decrease if no chelating agent is added to the medium (Dixon, 1985).

Plant growth regulators

The application of plant growth regulators to the culture medium is necessary for the successful induction of microspore embryogenesis. Auxin and cytokinin are the two most extensively used growth regulators in the anther culture of a wide range of plant species. In the anther culture of Poaceae and Brassicaceae 2,4-dichlorophenoxyacetic acid (2,4-D) is usually applied (Bishnoi *et al.*, 2000). Mitykó *et al.* (1996) used 2,4-D and kinetin to induce haploid embryo formation in the anther culture of *C. annuum*, and the same growth regulators were also used by Metwally *et al.* (1998) to produce haploid plantlets from the anther culture of *Cucurbita pepo*. Vigorous green plantlets are regenerated from microspore culture of *H. vulgare* in the presence of auxins such as indoleacetic acid (IAA) or naphthaleneacetic acid (NAA) (Castillo *et al.*, 2000).

Kinetin and benzylamino purine (BAP) are two cytokinins that promote shoot regeneration from within callus derived from *Oryza sativa* anther culture (Bishnoi *et al.*, 2000). Another type of cytokinin, zeatin, was found to be more effective compared to thidiazuron (TDZ) in promoting shoot development from within anther-derived callus of *L. usitatissimum* (Chen *et al.*, 1998a).

Although the application of growth regulators are found to enhance microspore embryogenesis in most species, their removal the from culture medium coupled with simultaneous lowering of the sucrose concentration resulted in the initiation of embryogenesis or shoot organogenesis in the anther

culture of *S. columbaria* (Romeijn and Lammeren, 1999). These observations support the assumption that the requirement of exogenous auxin and cytokinin depends on their endogenous level within the anther (Reinert and Bajaj, 1977).

Carbon sources

Sucrose is the most widely used polysaccharide in tissue culture, although other sources of carbon such as glucose, maltose and fructose are sometimes applied depending on the type and purpose of the programme (Trottier *et al.*, 1993; Coumans and Zhong, 1995; Kiviharju and Pehu, 1998).

Reinert and Bajaj (1977) suggested that the normal level of sucrose in most anther culture systems was 2 – 4%. However, Metwally *et al.* (1998) reported that a high level of sucrose (15%) produced more plantlets in *C. pepo* anther culture compared to lower concentrations. In contrast, Zhong *et al.* (1995) reported an increase of embryo germination in *H. annuus* anther when cultured on successive media with decreasing sucrose concentrations.

In microspore culture of *H. annuus*, Coumans and Zhong (1995) found that maltose showed a slightly superior effect over sucrose, and over different combinations of sucrose, glucose and fructose. Maltose proved to be better than sucrose as a carbon source in the anther culture of *Avena sativa* and *A. sterilis* (Kiviharju and Pehu, 1998). The addition of 3% maltose into the medium produced more vigorous plantlets in *H. vulgare* microspore culture (Castillo *et al.*, 2000). Similarly, Bishnoi *et al.* (2000) obtained a high frequency of shoot regeneration from within anther-derived callus in *O. sativa* anther culture by adding 3% maltose into agarose-solidified MS medium. The effect of maltose as a sucrose substitute, however, seems to be genotype dependent in *H. vulgare* anther culture (Trottier *et al.*, 1993). These observations suggest that the effect of the carbon source may be osmotic rather than a response to the carbohydrate source. It is known that different sources of carbon produce different osmotic potential in the medium due to differences in their molecular weight. The osmotic potential of the medium is a key factor for the uptake of water and other constituents by cultured anthers, thus affecting the success of androgenesis.

2.4.2.6 Pre-treatment of anthers

Various types of anther pre-treatment have been found to improve microspore embryogenesis in a wide range of species. These includes low temperature (Cistué *et al.*, 1998; Kiviharju and Pehu, 1998; Immonen and Anttila, 1999; Bishnoi *et al.*, 2000), high temperature (Kiviharju and Pehu, 1998) and mannitol starvation (Hoekstra *et al.*, 1997; Cistué *et al.*, 1998) or combinations of these.

A low temperature pre-treatment of floral buds or isolated anthers may enhance embryogenesis, even if donor plants are not initially grown at low temperatures (Palmer and Keller, 1997). For example, in *N. tabacum* Nitsch (1974) reported 58% embryoid formation if floral buds were pre-treated with a low temperature at 5°C for 72-h. In contrast, only 21% of anthers produced embryoids if floral buds were kept at 21°C for the same period. A significant increase in green plant regeneration is achieved in anther culture of spring and winter rye cultivars when anthers are subjected to a cold (4°C) pre-treatment for 2 - 4 weeks before being cultured (Immonen and Anttila, 1999). In anther culture of *O. sativa* var. *indica* Bishnoi *et al.* (2000) reported that cold pre-treatment (10°C) for 10 days was effective to induce microspore embryogenesis. Cold pre-treatment of anthers at 4°C had been found to be useful in anther culture of *Vitis vinifera* (Bensaad and Hennerty, 1996), *Secale sereale* (Immonen and Anttila, 1999) and triticale (× *Triticosecale*) (Immonen and Robinson, 2000). In *H. vulgare*, however, better results were obtained with mannitol starvation instead of low temperature pre-treatment (Cistué *et al.*, 1998). Pre-treatment of anthers in a starvation medium supplemented with mannitol was also found to be effective for androgenesis of *T. aestivum* (Indrianto *et al.*, 1999).

The importance of cold pre-treatment is attributed to the effect of temperature on tissue metabolism. At low temperatures the rate of metabolism slows down due to a decrease in enzyme activities. Subjecting the tissue to normal *in vitro* conditions may trigger the tissue to start out a new metabolic pathway. Nitsch (1974) suggested that cold pre-treatment influenced the first

division of the microspore, and as such two identical nuclei are formed rather than one vegetative and one generative.

Sometimes, pre-treating the anthers with high temperatures before culture initiation may enhance androgenesis. For instance, exposing *B. compestris* (Keller and Armstrong, 1979) and *B. oleracea* var. *italica* (Arnison *et al.*, 1990) anthers to temperature of 35ºC for 24-h before culturing them at 25ºC was reported to promote microspore embryogenesis. In addition, heat pre-treatment at 32ºC for 5 days given to isolated anthers of *Avena sativa* and *A. sterilis* was found to be the best for embryo induction (Kiviharju and Pehu, 1998). These stress pre-treatments of anthers are of critical importance for blocking gametophytic development and for triggering pollen embryogenesis in competent microspores (Touraev *et al.*, 1997).

2.4.2.7 Liquid versus solid media

In addition to the nutrient composition, the physical properties of the medium have to be considered before starting the culture. Agar-solidified media have been extensively used in many anther culture systems but the use of liquid mediua is now also becoming popular, particularly in microspore culture. Androgenesis in *T. aestivum* (Trottier *et al.*, 1993) and *H. vulgare* (Trottier *et al.*, 1993; Cistué *et al.*, 1998) involved the use of liquid media. Meanwhile, microspore embryogenesis in anther culture of *O. sativa* (Bishnoi *et al.*, 2000; Aryan, 2002) was successfully induced on a solid medium. However, neither solid nor liquid media were found to induce androgenesis in anther culture of *S. formosa* (Tade, 1992).

Another way of improving the yield of microspore-derived plants has included culture in a liquid medium supplemented with Ficoll-400™, a non-ionic copolymer of sucrose and epichlorohydrin, which functions as a buoyancy increasing component to allow anthers and calli to float. The detrimental anaerobic conditions of the liquid media are believed to cause the reduction in plant regeneration since anthers and calli developed under the medium surface. However, the results have not been consistent among studies or with different

species or cultivars. The addition of Ficoll-400™ to the liquid medium increased the number of embryos and green plants regenerated in anther culture of *H. vulgare* (Cistué *et al.*, 1998). With *T. aestivum*, Zhou (1992) found that Ficoll-400™ significantly reduced callus production but increased the percentage of regenerable calli and the ratio of green:albino plants. Immonen and Robinson (2000) reported that Ficoll-400 promoted a three- to four-fold increase in induction for the triticale (× *Triticosecale*) cultivar Bor 96151, but for the cultivars Modul and Wintri the induction either remains the same or decreased. The promotive effect of Ficoll-400™ is due to its ability to improve the density, viscosity and osmolality of the medium (Zhou *et al.*, 1992).

A new approach in anther culture is the use of a double-phase medium. The anther is grown in a thin layer of liquid medium, which is on the top of solid agar in the agar plate. This method has proven to be useful in the anther culture of triticale (× *Triticosecale*) (Immonen and Robinson, 2000).

2.5 Polyploidy induction using antimitotic chemicals

2.5.1 The use of colchicine and oryzalin

Colchicine or acetyltrimethylcolchicinic acid or (S)-N-(5,6,7.9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo (a) heptalen-7-yl) acetamide (Figure 1A), an alkaloid substance extracted from the roots of *Colchicum autumnale*, is a spindle inhibitor and acts on the dividing cells of plants (Jensen, 1974). Colchicine is the most reliable and widely used chemical in chromosome doubling of most plants (Wan *et al.*, 1989; Griesbach, 1990; Hamill *et al.*, 1992; Smith and Hamill, 1996; Takamura and Miyajima, 1996; Zhao *et al.*, 1996; Pinheiro *et al.*, 2000; Azhar *et al.*, 2002). Colchicine applications have proven to be useful in inducing polyploidysation and enhancing the agronomic performance of crop plants. Griesbach (1990) found better floral characteristics in *Anigozanthos* hybrids as the result of colchicine application. Colchicine-treated plants had larger, fuller and flatter flowers, as well as taller inflorescences and a stouter growth than untreated plants. Besides its improve horticultural merit, colchicine-treated *Anigozanthos* was fertile. A significant improvement in rhizome weight and knob

size of tetraploid *Zingiber officinale* following the treatment of rhizome segments (seed pieces) with 0.5% colchicine for 2 hours is reported by Smith and Hamill (1996). Polyploidization using colchicine also represents a useful method for the commercial breeding of *Cyclamen persicum* (Takamura and Miyajima, 1996) where the petals of colchicine-treated plants were larger and had a greater ability to accumulate chalchone, hence showed deeper colour than their diploid relatives.

Regardless of its promotive effects on the economic values of crop plants, colchicine is harmful to human beings, and in some cases shows undesirable mutagenetic activity in plants. Wan *et al.* (1989) have reported side effects of colchicine such as sterility, abnormal growth and morphology due to asynchrony of cell divisions. Chromosome losses or rearrangements and gene mutations caused by colchicine are reported in flax, *H. annuus*, barley and cotton (Lucket, 1989). The occurrence of chimaeras in doubled-haploid *Gerberra jamesonii* following colchicine treatments is reported by Honkanen *et al.* (1991).

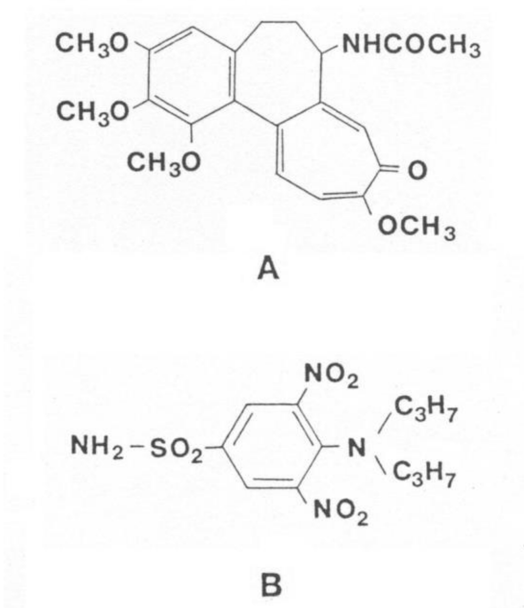

Figure 1.
Chemical structure of colchicine (A) and oryzalin (B).

Oryzalin (Figure 1B) or 3,5-dinitro-N^4,N^4-dipropylsulfanilamide, which was previously developed as a herbicide (Morejohn *et al.*, 1987) has also been

reported to inhibit mitotic activity and proved to be useful to induce chromosome doubling in many plant species such as *Z. mays*, *Malus domestica*, *G. jamesonii*, *Actinidia deliciosa* and *Lilium longiflorum* (Morejohn *et al.*, 1987; Wan *et al.*, 1991; Bouvier *et al.*, 1994; Tosca *et al.*, 1995; Chalak and Legave, 1996; Takamura *et al.*, 2002). In *Nicotiana plumbaginifolia,* Verhoeven *et al.* (1990) reported that the inhibition of spindle formation was stronger with oryzalin than with colchicine, which resulted in a more efficient accumulation of metaphases with well-scattered chromosomes. Oryzalin proved to be a more efficient chromosome-doubling agent than colchicine in potato cell suspension culture (Ramulu *et al.*, 1991). Following from this, Wan *et al.* (1991) apparently did not make a direct comparison between oryzalin and colchicine, but claimed that oryzalin very effectively induced chromosome doubling in anther-derived callus of maize but severely inhibited the growth of regenerable callus and plant regeneration.

It is clear that the effect of colchicine and oryzalin in inducing polyploidization varies with different plant species. Therefore, it would be useful to test the effectivity of both these antimitotic chemicals in inducing tetraploid *S. formosa.*

2.5.2 Methods of application

While colchicine is water soluble, oryzalin must be dissolved in dimethyl sulfoxide (DMSO) before use. Both concentration and time taken for action need to be taken into account. For prolonged treatment, a low concentration is required whereas for a short treatment, a high concentration is used. Many workers have reported the use of colchicine at a range of concentrations of periods of 0.01 to 2.0% for 1 hour to 7 days incubation, depending on the plant materials used (Hamill *et al.*, 1992; Kumashiro, 1992; Smith and Hamill, 1996; Takamura and Miyajima, 1996; Bishnoi *et al.*, 2000; Azhar *et al.*, 2002). In contrast, oryzalin was applied in much lower concentrations than colchicine, ranging from 0.001 to 0.02% for 3 hours to 3 days (Wan *et al.*, 1991; Tosca *et al.*, 1995; Takamura *et al.*, 2002). The use of very low concentrations of oryzalin is due to its stronger

inhibition of spindle formation compared to colchicine. In recent literature (Hamill *et al.*, 1992; Smith and Hamill, 1996; Azhar *et al.*, 2002; Broughton *et al.*, 2002), 2 – 4% (v/v) DMSO has been used as an effective carrier in colchicine application. DMSO greatly increases the permeability of cell walls and thereby improves the penetration of colchicine or oryzalin into the plant tissues, thus improving its action on the dividing cells.

The method of application varies among plant species and depends upon the plant material used. In some plants seed treatment is the most effective and safest way, but in others, where seeds are not available, colchicine is applied by soaking the rhizome (Smith and Hamill, 1996), sucker (Hamill *et al.*, 1992; Azhar *et al.*, 2002) or tuber (Takamura and Miyajima, 1996) segments. In *C. vulgaris* (McCuistion and Wehner, 2002) and *Passiflora incarnata* (Bruner, 1998) shoot apex treatment at the seedling stage was most effective.

Treating the whole seed undergoing germination with colchicine solution will affect all parts of the seedling as well as the growing point, although the growing point is the only target and the part that must be affected if the plant is to continue growing with an increased number of chromosomes. A major weakness of the method is that the roots are affected and usually grow poorly or do not grow at all. In addition, the occurrence of cytochimaera is often associated with the immersion of seeds, shoot tips or cuttings in colchicine solution (Pierik, 1997). Takamura and Miyajima (1996) reported the detrimental effect of colchicine on seed germination of *C. persicum.* The effect worsened with increased concentration. *In vitro* tuber segments, on the other hand, responded selectively depending upon the concentrations and duration of the treatment. Long-term *in vitro* treatment with 5% colchicine impeded plant regeneration and/or flowering, whereas short-term treatment with 2% colchicine proved ineffective to induce polyploidysation.

The active growth state of the plant is a key factor for antimitotic chemicals to work (Bruner, 1998). In contrast, the vegetative meristem is difficult to induce, and frequently results in mixoploid chimeras (Simmonds and Smart, 1979). Therefore, in breeding programmes where colchicine or oryzalin is used to double

the chromosome number, it is better to apply these compounds as soon as the seedling emerges from the seed rather than to the vegetative meristem.

It is still necessary to determine if the offspring are actually tetraploid. This is important because, as previously indicated, the application of antimitotic chemicals may also have a detrimental effect on plant growth. Polyploidysation can be confirmed through microscopic examination by directly counting the number of chromosomes or by assessing morphological characteristics such as stomatal size and density, leaf area and thickness, microspore size and flower size; or by using a flow cytometry method. Flow cytometry is an accurate and rapid method adapted to determine the ploidy level in plant tissues by measuring the nuclear DNA content in living cells. There has been increased use of flow cytometry in determining ploidy levels in a wide range of plant species (Sgorbati *et al.*, 1986; Dickson *et al.*, 1992; Tosca *et al.*, 1995; Husband and Schemske, 1998; Zonneveld and Iren, 2000; Azhar and Rusli, 2000a, 2000b).

2.6 Embryological development in sexual reproduction of legumes

Embryology is defined as a study of the developmental process in anthers and ovules leading to the formation of seeds (Prakash, 1987). The embryological process is a series of developmental processes leading to the formation of male and female gametes, pollination, fertilisation, and embryo formation and maturation. This process is an important factor determining seed production because an abnormality at any stage of the development may lead to the failure of reproduction in a plant species.

There is considerable embryological literature on the leguminous plants to which *S. formosa* belongs. However, despite botanical and agronomic studies (Thompson, 1990, 1993; Jusaitis, 1994), there is no adequate embryological data on this species. The importance of embryological information is not only in the evaluation of taxa (Prakash, 1987; Tsou, 1997; Floyd *et al.*, 1999) but also in the development of plant breeding programmes. Embryological data provides valuable information on many aspects of sexual reproduction as well as histology and developmental sequences in anthers and ovules leading to seed formation in

plants (Prakash, 1987). Embryology has a great potential to promote understanding of the breeding strategies of plant species, either using *in vitro* or *in vivo* techniques.

2.6.1 Anther wall and microsporogenesis

As with many other angiosperms, the anthers of legumes are generally tetrasporangiate, although bisporangiate anthers are seen in a few species (Prakash, 1987). Prakash and Chan (1976) and Lim and Prakash (1997) reported that the anther wall of *G. max* and *Psophocarpus tetragonolobus* are typically dicotyledonous. In addition to the epidermis, which is not part of the wall in the morphological sense, the anther wall at the mother cell stage consists of a one-layered endothecium, a two- or three-layered middle layer and a one-layered secretory tapetum. At the time of dehiscence, the middle layer and tapetum degenerate leaving only the epidermis and endothecium.

The occurrence of callose deposition in the microspore mother cell wall at the initiation of meiosis seems to be a universal trait in legumes (Prakash, 1987). After normal meiosis, the pollen mother cell undergoes simultaneous cytokinesis resulting in microspore tetrads that are generally tetrahedral. The microspores separate from each other and enlarge by the time of callose dissolution (Prakash and Chan, 1976). In *P. tetragonolobus* the mature pollen grains are triporate, two-celled, and show a reticulate exine layer (Lim and Prakash, 1997). Anther dehiscence was by longitudinal slits in the reptum of *G. max* (Prakash and Chan, 1976) resulting in an opening on the anther wall through which pollen grains were dispersed.

2.6.2 Ovule and megasporogenesis

As commonly observed in leguminous plants, the ovules are bitegmic and crassinucellar with a zigzag mycrophyle. A wide range of ovule orientation is found in leguminous plants such as hemianatropous to anatropous in Mimosoideae, anatropous to campylotropous in Caesalpinioideae and anatropous, hemianatropous, amphitropous or campylotropous in Papilionoideae (Prakash,

1987). Outer and inner integuments, each of two layers, enclose the nucellus. In *G. max* (Prakash and Chan, 1976) and *P. tetragonolobus* (Lim and Prakash, 1997) the outer integument developed more quickly than the inner integument and completely covered the nucellus at the end of megasporogenesis.

The multicellular archesporium is widespread in leguminous plants (Prakash, 1987) but Prakash and Chan (1976) found only a single-celled archesporium in the ovule of *G. max*; this is a common feature of many angiosperms (Johri *et al.*, 1992). The hypodermal archesporium of legumes follows the common trait in angiosperms (Johri *et al.*, 1992) although in some species the sub-hypodermal archesporium is also found (Prakash, 1987). As with normal ovule development in Poaceae (Brown and Emery, 1958), a single megaspore mother cell in legumes undergoes meiosis to form a linear tetrad of four megaspores. Usually, the three micropylar megaspores degenerate simultaneously leaving only the chalazal megaspore that functions (Prakash, 1987).

At its mature stage, the female gametophyte is bordered by the inner integument at the sides, a single-layered epidermis at the micropylar end, and the hypostase at the chalazal region (Prakash, 1987). However, in *P. tetragonolobus* Lim and Prakash (1997) found that the endothelium is not differentiated. This finding is contradictory to most angiosperms that are reported to develop an integumentary tapetum or endothelium from the innermost layer of the integument in contact with the embryo sac (Johri *et al.*, 1992).

2.6.3 Pollination and fertilisation

The first step in germination is the expansion of the pollen grain by the absorption of liquid from the moist surface of the stigma and the protrusion of the intine through a germ pore. The small tubular structure of the pollen tube, which arises in this way, continues to elongate and travel down the tubular stylar canal. As with most angiosperms, the pollen grains of legumes are generally monosiphonous (Prakash and Chan, 1976), i.e. only a single pollen tube emerges.

This is different from members of the Malvaceae, Cucurbitaceae and Campanulaceae that have polysiphonous pollen grains on germination.

The stigma is believed to play an important role in the germination of pollen. It is not only the landing and germination platform for pollen grains, but also functions in recognition and in compatibility or incompatibility reactions (Maheshwari, 1950). In some plants the stigma releases exudate for protection against excessive desiccation, and this also functions as a germination medium for compatible pollen grains. In others, the stigma is dry and covered by a pellicle of external protein that becomes hydrated following pollen adhesion to the papillae. The cuticle of the stigma is dissolved, and the pollen tube enters the transmitting tissue of the stigma (Johri *et al.*, 1992). The mechanical removal of the stigmatic cuticle by tripping the flower or rubbing the stigma several times using a finger tip, has proven effective in increasing the pollination rate in *S. formosa* (Jusaitis, 1994).

During fertilisation the pollen tube reaches the nucellus through the micropyle (Prakash, 1987); this process is called porogamy. On the other hand, *Pistacia* sp. (Anacardiaceae) and members of the Betulaceae and Juglandaceae showed pollen tube penetration through the chalaza (chalazogamy) (Johri *et al.*, 1992). As with most angiosperms, the entry of the pollen tube into the embryo sac in legumes is through one of the synergids. The other synergid degenerates soon after fertilisation takes place (Prakash, 1987; Lim and Prakash, 1997). However, in *Indigofera annaephylla* the pollen tube enters the embryo sac between the egg and one synergid without destroying the synergid (Desphande and Untawale, 1971).

Double fertilisation, which occurs in a wide range of angiosperms, is also observed in the majority of legume genera (Prakash, 1987). Johri *et al.* (1992) described the process of double fertilisation in angiosperms to be indicated by the fusion of one male gamete (sperm) with the egg cell (syngamy), while the other male gamete fuses with the secondary nucleus or the two polar nuclei (triple fusion). At the time of fertilisation, the male gamete is at the late telophase of mitosis, while the egg nucleus is in the aging or maturing phase.

2.7 Conversion of vegetative to reproductive growth

It has been known that floral initiation is controlled by environmental factors, particularly photoperiod and temperature. Many plants show different responses to different photoperiod and temperature regimes and enter the reproductive stage under specific combinations of these two factors. Depending on their responses to photoperiod, the plants are classified into three categories, i.e. short-day, long-day and day-neutral plants. In addition to photoperiod, certain plants require an exposure to low temperature regimes before they start to produce flowers; this is known as vernalisation.

Regarding photoperiod requirements, the work of Yusuf (2002) showed that floral initiation in *S. formosa* was not dependent on photoperiod. This finding confirmed the report of Williams (1996) that *S. formosa* flowered all year round, although floral initiation on plants grown under longer photoperiods was faster than those grown under shorter photoperiods.

There have been some theories developed to describe the endogenous control of floral induction. These include the florigen concept, nutrient diversion hypothesis and the involvement of plant hormones (Bernier, 1988). According to the florigen concept, the effect of a suitable photoperiod mediated by phytochrome may result in the synthesis of a hypothetical floral stimulus named florigen. This hypothetical hormone is formed in the leaves, including the cotyledons, and is transported to the apical meristem where it evokes changes committing the plant to subsequent flower formation. During the evocation stage, a rise in RNA synthesis has been observed as the earliest change in the apical meristem metabolism (Bernier, 1971; Seidlova, 1974). In addition, Jacqmard *et al.* (1972) reported an increase in the amount of total protein in the apical meristem. A rise in mitotic activity is another early event following the arrival of the floral stimulus in the meristem (King, 1972). After the evocation stage, DNA synthesis is stimulated and a further rise in mitotic activity occurs. These processes are involved in the production of cells that will give rise to floral primordia.

Florigen has yet to be isolated from plants although there is much evidence for its existence in plants. Since no known chemical has been found to have a clear promotive effect on flower initiation in all plants so far, florigen is hypothesised to be an unknown hormone with a specific role in the control of flowering.

Many factors that influence photosynthesis and/or assimilate availability are also found to control flower initiation in certain plants. Sachs and Hackett (1983) postulated the “nutrient diversion” hypothesis. This hypothesis states that whatever the nature of involved factors, it is a means of modifying the source-sink relationship within the plant in such a way that the shoot apex receives a higher concentration of assimilates than under noninductive conditions. In long-day plants, Baysdorfer and Robinson (1985) and Britz *et al.* (1985) found that the proportion of photosynthates retained as starch was much lower and assimilate export was higher than in short-day plants.

Under *in vitro* conditions, sucrose or glucose application promotes flowering in some plants, even under noninductive conditions. The optimal sugar concentration for flower production by nonphotosynthetic explants is generally far above that for vegetative formation (Bernier *et al.*, 1981). Sucrose feeding causes earlier flower initiation in *Brassica campestris* (Friend *et al.*, 1984). Thus, the effect of sucrose is specifically on flowering rather than simply through a general promotion of growth.

Parts of floral evocation and morphogenesis can be produced by the application of various chemicals including plant hormones. This developmental step is believed to occur only when all other factors are present in the apex at appropriate concentrations and times. While assimilates and plant hormones are generally present in most plants, some of these compounds may be absent or present in sub- or supraoptimal amounts (Bernier, 1988).

Of all plant hormones that have been recognised so far, gibberellins are the most extensively studied in relation to flower initiation in many plant species. However, their role in controlling flower initiation is a controversial matter. Gibberellin can overcome the suppression of bolting and flowering caused by

growth retardants in *Arabidopsis thaliana.* This suggests that gibberellin is the primary controlling factors of flowering in this species. However, in spinach, growth retardants suppress bolting and reduce gibberellin levels significantly but leave flowering unaffected. In addition, gibberellin cannot induce flowering in all rosette plants, and many caulescent species do not flower after gibberellin treatment. In other species, however, including *Pharbitis nil, Impatiens balsamina, Chrysanthemum morifolium, Bryophyllum calycinum, Cordyline cannifolia, V. vinifera* and conifers there is strong evidence that gibberellins are part of the promoters of flowering. Gibberellin is found to be clearly inhibitory in some angiosperm such as *Fragaria ananassa*, *Fuchsia triphylla, Poa pratensis, C. sinensis, L. esculentum* and *M. domestica* (Bernier, 1988).

There are some reasons for the complexity of the role of gibberellin in flower initiation, i.e. the effectiveness of gibberellins is different depending on species (Looney *et al.*, 1985; Pharis *et al.*, 1987). In some plants the gibberellin effect is enhanced or even only detectable when gibberellin is associated with an adjunct treatment such as water stress or root pruning (Pharis and Ross, 1986). The timing of gibberellin application is a critical factor (Bernier *et al.*, 1981) and gibberellin responses may be greatly influenced by growing conditions with the effects generally much greater under long photoperiods than under short photoperiods or at moderately low temperatures than at higher temperatures (Metzger, 1985).

To summarise, it can be suggested that understanding the factors affecting floral initiation may result in a better prediction of the conversion time from the vegetative to the reproductive stage in *S. formosa.* This is particularly useful in the induction of polyploidy using antimitotic chemicals such as colchicine or oryzalin because the application of such chemicals will only be effective during the vegetative growth stage. It is important to note that once conversion to floral apices has occurred, the application of colchicine or oryzalin will no longer be effective.

2.8 Conclusion

The development of male-sterile flowers has become a critical issue in the commercialisation of many flowering plants; especially those that are marketed as cut flowers. The occurrence of male-sterility and the potential of ploidy manipulation, which has widely been recognised in many plants, is a valuable aspect that merits investigation to produce pollenless progeny in *S. formosa.*

Many authors have investigated the success of ploidy manipulation either in the form of tetraploid production or haploid and doubled-haploid regeneration. Any aspect related to the use of the technique has also been very well documented. However, despite the success in many cereals, particularly *T. aestivum*, *S. cereale*, *O. sativa*, *S. bicolor* and *H. vulgare*, the investigation on the regeneration of haploid plants via anther culture in legumes has shown limited success. Similar results have also been shown in the induction of tetraploids using antimitotic agents. This difficulty could be attributed to the problems with ploidysation of the legume tissue as proposed by Van Doorne *et al.* (1995).

Knowledge about the embryology of sexual reproduction can provide prediction of reproductive success. The development of the anther wall and ovule, as well as micro- and megasporogenesis followed by pollination and fertilisation, which are critical in maintaining the continuity of the population, are of major importance as fundamental aspects of plant breeding. These are all crucial aspects that deserve detailed investigation, particularly in *S. formosa.*

Although floral initiation in *S. formosa* is not photoperiod-dependent, the understanding of the exact time of conversion from the vegetative to the reproductive stage is a key factor for the successful application of antimitotic agents. This is because polyploidy induction may only occur if these chemicals are given to plants during vegetative growth. Therefore, understanding the conversion from vegetative to reproductive growth in *S. formosa* is a key factor that merits investigation.

CHAPTER 3
THE SWAINSONA FORMOSA PLANT

3.1 Introduction

Swainsona formosa (Figure 2), a legume native to Australia (Williams and Taji, 1992), belongs to the subfamily Papilionoideae of the family Fabaceae. It grows mainly in arid regions (Figure 3) of southern and central Australia (Williams and Taji, 1987, 1991) in South Australia and also occurs in New South Wales, the Northern Territory and Western Australia (Kirby, 1996a, 1996b), particularly on alkaline soils of areas receiving extra water, such as creek or lake margins (Bindon, 1996).

Figure 2.
All-red variety of *S. formosa* in flower. Bar = 2 cm.

Previously *S. formosa* was named *Clianthus formosus* (Greek, *kleios* = glory, *anthos* = flower and *formosus* = beautiful) by Ford and Vickery in 1950. However, following the revision of the genus *Swainsona,* J. Thompson, a botanist at the National Herbarium of New South Wales, claimed that *C. formosus* has closer affinity with Western Australian species of *Swainsona* than with the New Zealand-based genus of *Clianthus.* Therefore, she reclassified *C. formosus* as *Swainsona formosa* (Thompson, 1990, 1993).

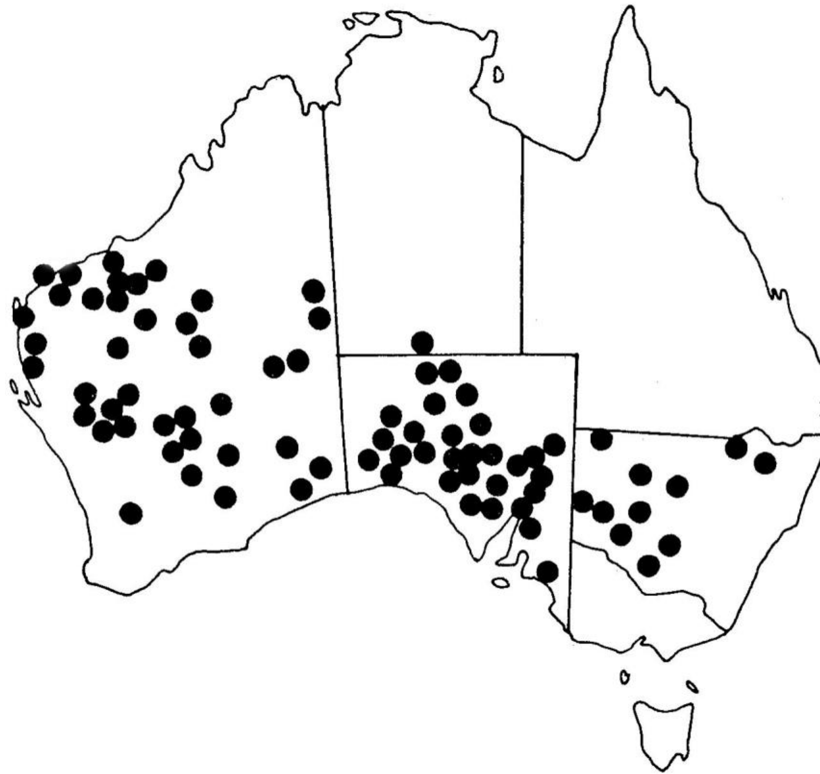

Figure 3.
Distribution of *S. formosa* in Australia (Thompson, 1993).

Growing naturally, *S. formosa* grows as a prostrate annual with long trailing stems radiating up to 2 m from the main woody root (Williams and Taji, 1991). The seedling generally develops a strong central stem that grows upright to an average height of 28 - 30 cm, and even up to 50 cm (Mitchell and Wilcox, 1994). Two prostrate laterals develop at the base and continue to grow until the central stem produces flowers and ceases to grow. If the central stem is supported using an upright trellis, it continues to climb upwards and produces cluster of flowers at every node. Within 105 days of planting, the stem will achieve 2 m in length, and produce up to 28 clusters on a trellised runner. Staked lateral runners grow rapidly, producing flowers at each node rather than side branches. When these lateral runners reach a height of 30 cm or more, additional laterals appear at the base of the plant. When *S. formosa* is growing as a pot flower, the best trellising and pruning system is two plants in a pot with three runners staked from each plant, i.e. the central stem and the first two laterals (Barth, 1990).

As with some other members of the Papilionoideae, the whole plant is covered with long soft hairs. The leaves consist of 9 - 17 grey-green oval leaflets, 1 - 3 cm long, fringed with grey hairs, and borne alternately on the axis (Bindon, 1996).

Plants grow best in full sun (Williams, 1996) and need high light intensities for flowering (Kirby, 1996b). Yusuf (2000) found that there was a strong correlation between light intensity and flower production. Plants grown under light intensities of 600 µmol $m^{-2}s^{-1}$ flowered earlier than those grown under higher light intensities (950 µmol $m^{-2}s^{-1}$) but the number of flowers was the same. Meanwhile, when the light intensity was reduced to 140 µmol m $m^{-2}s^{-1}$ no flowers were produced. During the middle of the day in midsummer, when light intensity is very high, Kirby (1996b) suggested a provision of 70% shade to reduce moisture stress without a reduction in flowering.

Flowering of *S. formosa* is not affected by day length and plants will flower all year round (Williams, 1996). However, a longer photoperiod (16 hours per day) was required for faster flower appearance, and a neutral photoperiod (12 hours per day) produced more flowers per plant (Yusuf *et al.*, 2002).

Although *S. formosa* is a temperate plant, it does not tolerate a high temperature regime (Barth, 1990) unless sufficient water is provided (Kirby, 1996b). The optimum temperature is 32.5 ± 2.5ºC, and plants will grow slowly if daily temperature is below 20ºC (Kirby, 1996a). Cold night temperatures (12 – 15ºC) will stop plant growth without causing any damage (Williams, 1996). A wide fluctuation in day and night temperatures will produce elongated plants (Barth, 1990), and warmer night temperatures usually result in more compact plants (Williams, 1996). Plants grown under temperature regimes of 18/30ºC (night/day) produced more flowers than those grown under 10/22ºC (night/day) or a constant temperature of 25ºC (Yusuf *et al.*, 2002).

S. formosa is highly susceptible to *Fusarium moniliforme,* a systemic wilt disease producing spores which are air-dispersed and easily spread once established (Barth, 1990). This plant is also reportedly prone to sudden collapse when fully grown and coming into flower, due to root disease (Williams and Taji,

1991). High humidity along with high temperature can also lead to grey mould (*Botrytis cinerea*) of the foliage and runners, unless the foliage is kept dry (Kirby, 1996a, 1996b). Meanwhile, the major insect pests of *S. formosa* are two-spotted mite, aphids, mealy bugs, thrips (Barth, 1990) and heliothis caterpillar (Kirby, 1996a, 1996b). A native butterfly (*Lampides boeticus*) is a serious pest of *S. formosa* plants grown outdoors during summer (Kirby, 1996a, 1996b).

3.2 The flower

It takes 10 - 12 weeks for *S. formosa* to flower when grown from seed under favourable conditions, and the flowers will be produced continuously over many months (Williams and Taji, 1991). A wide variation in flowering time is found in the wild, and some plants may take more than 16 weeks to start flowering. Kirby (1996a) reported that the time taken to induce flowering was temperature and season dependent. Plants growing in hot areas flower much more quickly than those growing in cold areas. In addition, it was found that plants growing in the autumn took longer to flower than those growing in the spring.

Mitchell and Wilcox (1994) described the *S. formosa* flower as a large, flag-like flower, up to 10 cm long, coloured red with a dark red or black boss (Figure 4A). Tade (1992) reported that the flower consists of five sepals (calyx) and a five-part corolla (petals) that are typical to legumes. Furthermore, Jusaitis (1994) observed that the petals incorporate a shiny black boss and a lower keel that houses the sexual organs. However, since *S. formosa* is out-crossing in nature (Williams and Taji, 1992), the flowers vary widely in colour as well as size (Figure 5). The inflorescences consist of clusters (umbels) of up to six flowers in a whorl at the end of short upright stalks (Williams and Taji, 1987). A well grown plant can produce up to 150 flower clusters (Williams and Taji, 1991). The peduncle may be up to 20 cm long (Jusaitis, 1994). Pods are narrow-elliptic-oblong and covered with dense hairs, 4 - 9 cm long, and contain numerous laterally flattened brown seeds (Figure 4B,C) (Bindon, 1996).

As in all legume flowers, the flower of *S. formosa* has ten stamens, which collectively constitute the androecium. The filaments of nine stamens are fused

and elevated as a single structure, whereas one stamen (the posterior one) remains separated. The pistil that elongates beyond the anthers is unicarpellate and the outer surface is covered by trichomes. The style is about half the length of the pistil and curves slightly backwards, towards the free posterior stamen, terminating at the stigma (Tade, 1992). Like many other legumes, the stigma of the *S. formosa* flower is covered by a stigmatic cuticle that, unless ruptured, prevents self-pollination from occurring (Jusaitis, 1994).

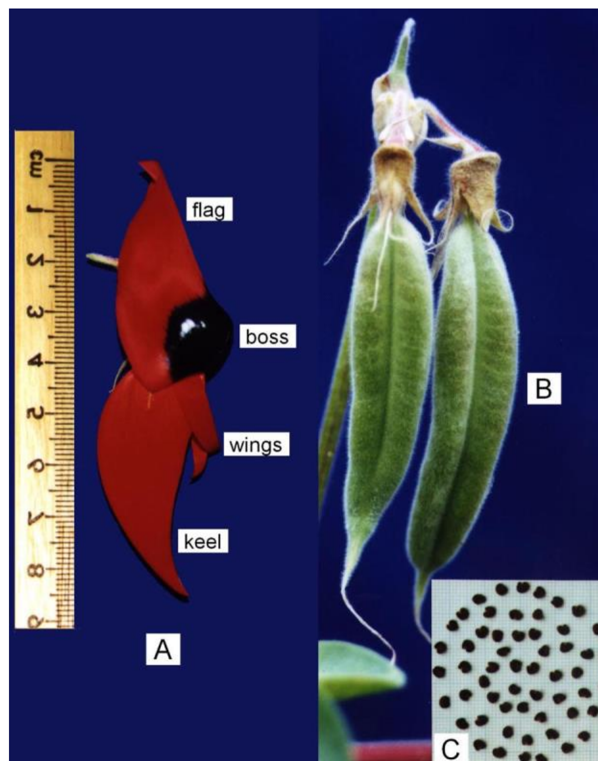

Figure 4.
Flower, pods and seeds of *S. formosa.* A, large, flag-shaped flower coloured red with black boss; B, narrow, elliptic oblong pods covered with dense hairs; C, laterally flattened brown seeds.

The vase-life of *S. formosa* flowers can reach 3 weeks in winter, although it may shorten to 7 - 10 days in hot conditions. At a constant temperature of 23ºC, most scapes remain attractive for 10 - 14 days (Kirby, 1996b). The vase-life of cut flowers of *S. formosa* could be increased by up to 50% by using a 4 mM silver

thiosulphate (STS) pre-treatment. When combined with pulsing for 18 hours in 4% sucrose solution and holding the flowers in base preservative solution containing 100 ppm stabilised chlorine plus 250 ppm citric acid without sucrose, the vase-life increased by up to 157% (Barth, 1990).

Figure 5.
Some variations in colour of *S. formosa* flowers. A, white-flowered; B, all-pale pink; C, pale pink with red boss; D, pink-flowered with darker boss; E, dark pink-flowered with darker pink boss; F, classic red-flowered with shiny black boss (Photographs B-D and F courtesy of Professor Acram Taji, UNE).

The use of seaweed concentrate has also proven to be effective in increasing the vase-life of *S. formosa* flowers. Plants sprayed with 1.75% (w/v) Natrakelp® (a seaweed concentrate) at 4 and 8 weeks after germination produced individual flowers that lasted 5 days longer than untreated plants. In addition, the vase-life

of flowers on runners with 7 leaves and 3 umbels increased by 3 days (Jones *et al.*, 1996).

3.3 Flower development

S. formosa is a monoecious plant, and both anthers and stigma are present in the lower keel of the flower. However, cross-pollination in this species is enhanced by the presence of a stigmatic cuticle that precludes pollen germination until the cuticle is ruptured and the receptive surface below the cuticle is exposed (Jusaitis, 1994).

Jusaitis (1994) reported that there were 7 stages of floral development in *S. formosa*; these took 17 days from bud initiation to anthesis (Figure 6). The seven stages are: stage 1, flowers tightly clustered in buds, petals completely enclosed by calyx; stage 2, tips of petals begin to protrude through the calyx showing the first sign of colour; stage 3, flowers oriented horizontally during the process of downward curvature of the pedicels; stage 4, pedicels had completed their downward curvature and the flowers are oriented vertically; stage 5, the flag began to reflex back from the keel; stage 6, the flag in half-reflexed horizontal position; and stage 7, the flag had fully reflexed and the flower was open.

The growth of *S. formosa* flowers is slow at the beginning, and most rapid after 2 months of germination (Tade, 1992). The length of the peduncle followed a sigmoidal growth pattern over the period of floral development, with maximal length being achieved by 19 - 20 days. During the first half of this period, the flowers remained at stage 1, but when the peduncle growth became linear, floral development accelerated quickly through stages 2 and 3 to reach stage 4. Floral development slowed at stage 4, while the peduncle continued to elongate. Flowers then passed through stages 5 - 7 as the peduncle elongation rate declined. Floral stages 5 and 6 were the most rapid, both occurring on the same day. Floral development was completed 17 days after the emergence of a floral bud, but peduncle growth continued slightly beyond that time (Jusaitis, 1994).

Anther dehiscence commenced at stage 5 and was completed by stage 6. This indicates that, for the breeding programme, emasculation should be carried

out during stage 5, when the anther is fully developed but has not dehisced. Following anther dehiscence, pollen fell to the bottom of the keel and accumulated immediately posterior to the stigma, in the region of the stylar brush hair. In addition to the presence of a stigmatic cuticle, these hairs also play an important role in promoting cross-pollination in *S. formosa* (Jusaitis, 1994). The production of nectar at the base of the filament tube commenced during stage 6, and by stage 7 each flower contained 80 - 100 µL of nectar (Barth, 1990).

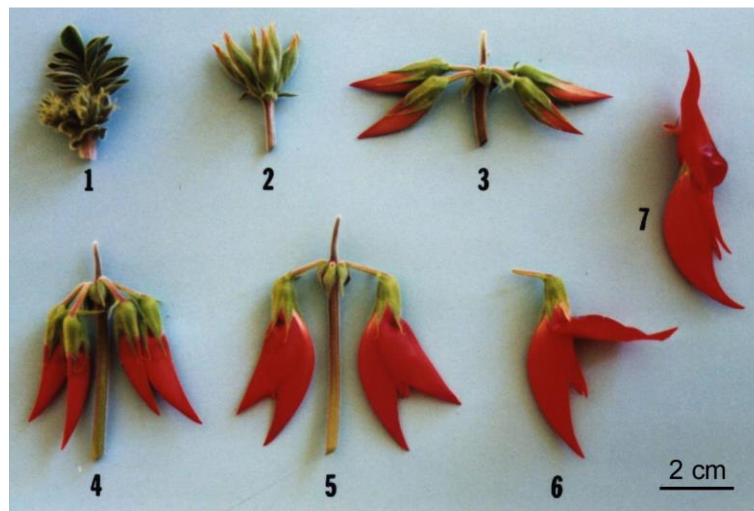

Figure 6.
The seven stages of floral development in *S. formosa* adapted from Jusaitis (1994).

It is obvious that *S. formosa* is one of the most popular flowering plants in Australia. This is not only because it is a native species and the floral emblem of South Australia, but also because of its potential use as a cut flower or hanging basket plants or flower. The brilliant colour, along with the unique shape of the flower, has made it an attractive ornamental plant. However, some floral characteristics need further improvement to support its commercialisation and to fulfil market interest. It is for these reasons that *S. formosa* was chosen to be the subject of this thesis.

CHAPTER 4
GENERAL MATERIALS AND METHODS

4.1 Introduction

The procedures used throughout the project are described in general in this chapter. Specific protocols for a particular topic are outlined in the specific chapters under relevant headings. All experiments were conducted using facilities available at the School of Rural Science and Agriculture and the School of Environmental Sciences and Natural Resources Management, University of New England, Armidale, NSW. These included the glasshouses, the Plant Biotechnology laboratory and the Microtechnique laboratory. The voucher specimen was deposited at the NCW Beadle Herbarium, University of New England (accession number NE79130).

4.2 Stock plant establishment

4.2.1 Seed germination

Seeds were soaked for 10 minutes in 1.0% NaOCl (obtained by diluting 25 mL commercial bleach (Super Strength Bleach™) containing 4.0% available chlorine, in 75 mL deionised water). They were then soaked for 10 seconds in just-boiled water before being rinsed with sterile deionised water. The sterile seeds were then soaked overnight in cool sterile deionised water. Within the next 24 hours, the swollen seeds were plated onto a moistened double-layer sterile filter paper in a Petri dish. The dish was then kept on a shelf in a culture room with a temperature of 23 ± 2ºC and with a 16-hour photoperiod.

4.2.2 Transplanting to Jiffy pots

Seeds germinated within 48 hours. The testa was removed soon after radicle formation to aid cotyledon development and to obtain a high growth rate and uniform seedlings (Figure 7A). Following germination, seedlings were transplanted into Jiffy pots (Jiffy-7™) that were prepared by soaking compressed

peat pots in water for 15 minutes. The seedlings were placed in a shaded glasshouse, with light intensity ranging from 100 to 170 µmol m^{-2} s^{-1} and with day/night temperature of 25/18ºC and relative humidity approximately 80%, to allow further growth.

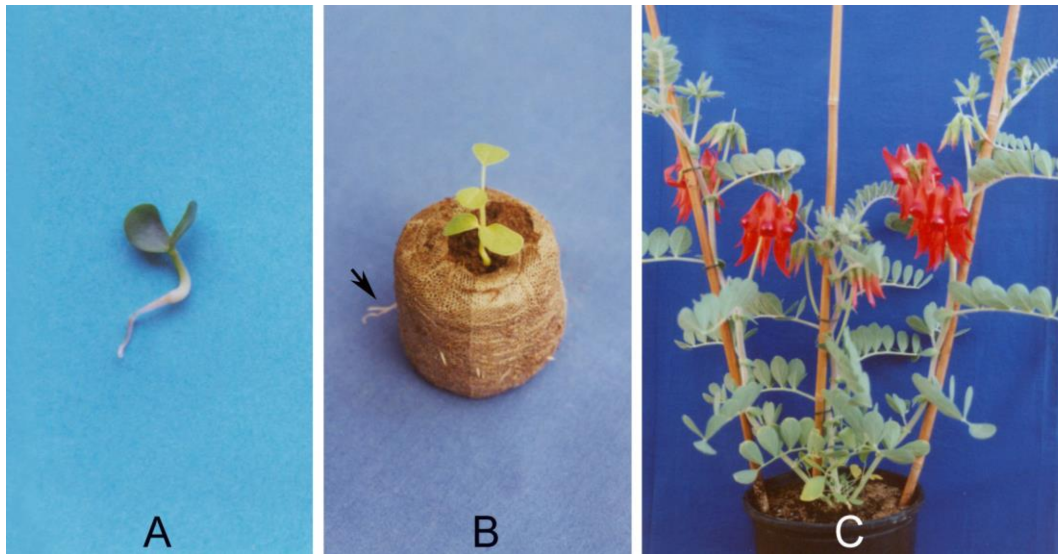

Figure 7.
Seedlings and mature plant used as the source of various experimental materials. A, one-week old seedling; B, seedling at 14-days in Jiffy pot (arrow indicates roots protruding from Jiffy pot); C, mature plant.

4.2.3 Transplanting to plastic pots and care of plants

Within 3 weeks after germination (14 days in Jiffy pots), the seedlings produced adequate root systems protruding from the base and the walls of the pots (Figure 7B). Seedlings were then transplanted to 20-cm pots (Pite-GRO 200 mm, 5 L capacity) at a density of one plant per pot. The growing medium consisted of sand and peat (3:1), thoroughly incorporated, wetted down, pasteurised at 80ºC for 30 minutes to eliminate pathogenic microorganisms and weed seeds, and left to cool until required. Once potted, the plants were kept in a temperate glasshouse with an average temperature range of 25 - 32ºC and light intensity ranging from 650 - 1200 µmol m^{-2} s^{-1}. Plants were watered once a day using an automatic watering system. Approximately 250 mL supplementary liquid fertiliser with trace elements, Hortico™ Aquasol™ (23% N, 4% P, 8% K, 0.05%

Zn, 0.06% Cu, 0.0013 Mo, 0.15% Mn, 0.06% Fe and 0.11% B), was applied weekly at a concentration of 1 g L^{-1}. After the plants had been four weeks in the new pots, a fungicide (Fongarid® 250WP with active ingredient 25% furalaxyl) was applied at a concentration of 1 g L^{-1} to eliminate root diseases such as *Phytopthora* sp. A slow release fertiliser, Nutricote® (16% N, 4.4% P, 8.3% K and 4% Ca), was applied at the time of transplanting and after 8 weeks of transplanting at a concentration of 2 g per pot for each application to ensure optimum nutrient supply. The release time of Nutricote® was approximately 90 days at a soil temperature of 35°C, and as such was replenished twice in the life of the experimental plants. A miticide, Omite® 300W (active ingredient 30% propargite), was applied at the rate of 1 g L^{-1} to kill the spider mite (*Tetranychus* sp.) as required. In addition, an insecticide Clear White Oil (active constituent 820 g L^{-1} petroleum oil) was applied at 0.2% (v/v) to kill mealy bug.

In order to obtain better plant growth and to allow better light penetration, any unwanted axillary branches were removed and only two branches growing close to the soil surface were left. Therefore, each individual plant consisted of one main stem and two axillary branches. These stems were staked using bamboo rods to support upright growth.

4.2.4 Hand-pollination

Flowers were hand-pollinated to aid in fertilisation and seed production. Flowers at anthesis were pollinated by rubbing the stigma several times with a fingertip covered with freshly shed pollen grains. Rubbing the stigma is important to remove the stigmatic cuticle to expose the receptive surface below (Jusaitis, 1994). Both ovule and pollen grains were from the same flower or from different flowers on the same plant.

A sufficient number of pods and seeds were produced from hand-pollinated flowers, and these were used for the next stock plant establishment.

4.3 *In vitro* work

4.3.1 Medium

Unless otherwise stated, the medium used in this experiment was B5 (Gamborg *et al.*, 1968). The composition of this medium was first developed for the suspension culture of soybean root cells, but is now widely used for *in vitro* culture of many legumes. To avoid chemical interactions leading to precipitation, and for ease of use, chemicals in the B5 medium were grouped as per Table 3 and stock solutions were prepared at 100x required concentrations.

Table 3.
List of the constituents and the amount needed for the preparation of 1 L of B5 basal medium from stock solutions.

Solutions	Chemicals	Stock (g L^{-1})	Amount per litre medium (mg)
A	$(NH_4)_2 \cdot SO_4$	1.34	134.00
B	KNO_3	25.00	2,500.00
C	$CaCl_2 \cdot 2H_2O$	1.50	150.00
D	$NaH_2PO_4 \cdot H_2O$	1.305	130.50
	H_3BO_3	0.003	3.00
	$Na_2MoO_4 \cdot 2H_2O$	0.0025	0.25
	KI	0.0075	0.75
	$CoCl_2 \cdot 6H_2O$	0.00025	0.025
E	$MgSO_4 \cdot 7H_2O$	2.50	250.00
	$MnSO_4 \cdot H_2O$	0.100	10.00
	$ZnSO_4 \cdot H_2O$	0.002	2.00
	$CuSO_4 \cdot 5H_2O$	0.00025	0.025
F	$FeSO_4 \cdot 7H_2O$	0.278	27.80
	Na_2EDTA	0.373	37.30
Vitamins	*myo*-inositol	1.00	100.00
	nicotinic acid	0.001	1.00
	pyridoxine-HCl	0.001	1.00
	thiamine-HCl	0.010	10.00
Sucrose	-	-	20,000.00

Ten millilitres of each stock solution was added with the solution being stirred constantly. The sucrose was added and dissolved, and the volume was made up to 1 L with distilled water. The pH of the medium was adjusted to 5.8 ± 0.02 with either 1 M NaOH or 0.5 M HCl using an Activon Model 210 pH meter. Where needed, the medium was solidified by the addition of 8 g L^{-1} of Bacto Bitek™ agar which was heated and dissolved prior to dispensing into the culture tubes and autoclaving at 1.1 kg cm^{-1} (103 kPa) at 121ºC for 20 minutes.

4.3.2 Explants

Materials used as explants were anthers taken from young floral buds containing microspores at different developmental stages (Figure 8). These buds were obtained from clusters on at least 5 different plants that were preselected randomly from glasshouse-grown populations. In order to obtain homogeneity among plant materials, all buds were taken from the 3rd to 5th clusters on each stem.

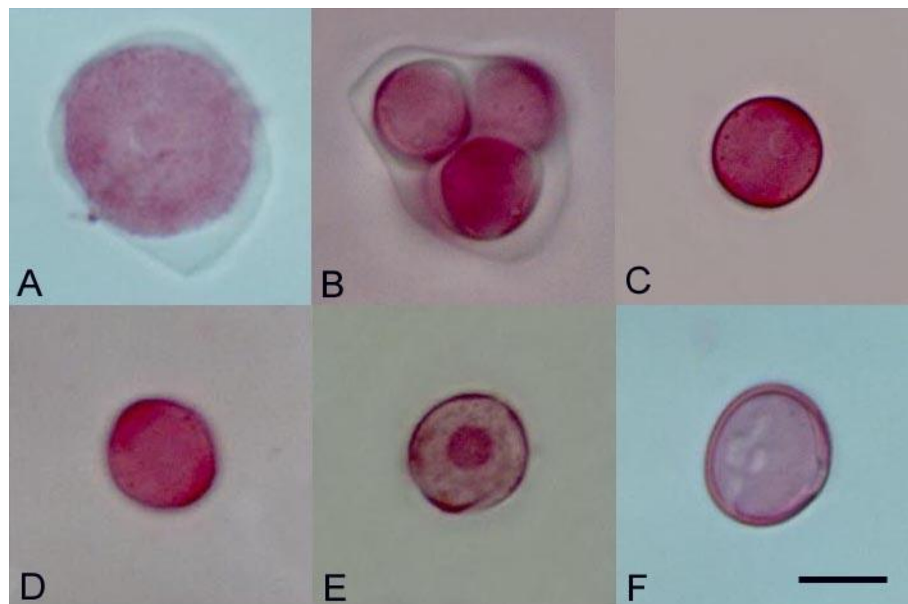

Figure 8.
Cytological stages of *S. formosa* microspores used throughout the experiments: A, mother cell; B, tetrad; C, early-uninucleate; D, mid-uninucleate; E, late-uninucleate; F, immature pollen grain. Bar = 10 µm.

Flower clusters with 5 - 6 flowers that were 1.3 – 1.6 cm long were collected during the day between 10.00 a.m. - 12.00 noon and surface-sterilised in 70% alcohol for 10 seconds followed by rinsing in sterile distilled water before being used. The flowers were separated from each other and only 5 flowers were used to make 5 replicates for each treatment.

The sepals and petals were carefully removed to expose the anthers, using clean and sterile forceps and scalpel. Anthers were separated from the filaments and cultured at a density of 10 anthers per culture flask. These 10 anthers were obtained from a single flower.

4.4 Microtechnique work

4.4.1 Collecting and fixing the specimen

Floral buds, flowers and shoot apices of different stages were collected from glasshouse-grown plants. These materials were fixed in a formalin-propiono-alcohol (FPA) solution for at least 48 hours until required. The FPA was prepared by mixing 90 mL 70% ethyl alcohol + 5 mL propionic acid + 5 mL formalin (Prakash, 2000).

The outer coverings of floral buds, flowers and shoots of easily manageable size were removed using clean forceps and scalpel and the anthers were left intact prior to introduction into the fixing solution. Small buds that were difficult to handle individually were directly fixed in a freshly prepared fixing solution. In order to obtain proper fixation, any air bubbles particularly those trapped within small buds were removed by gently squashing these buds using clean forceps. Trapped air bubbles may prevent the penetration of the fixative agent into the tissue. A non-fixed tissue may undergo further changes in cell chemistry and structure, while fixation is a process by which the cellular and structural elements are preserved in their natural conditions (Prakash, 2000).

4.4.2 Dehydration

Before dehydration the specimens were washed in 70% alcohol for 5 – 10 minutes. The specimens were dehydrated through a series of tertiary butyl

alcohol solutions (TBA) for 2 hours in each grade (‘50’, ‘70’, ‘85’, ‘95’ and ‘100’) and for 24 hours in pure TBA (with one change after the first 12 hours). The different grades of tertiary butyl alcohol were prepared as shown in Table 4.

Table 4.
The preparation of the tertiary butyl alcohol (TBA) series for specimen dehydration.

Constituents	Grade				
	‘50’	‘70’	‘85’	‘95’	‘100’
Distilled water	50	30	15	-	-
95% ethyl alcohol	40	50	50	45	-
Tertiary butyl alcohol	10	20	35	55	75
100 ethyl alcohol	-	-	-	-	25

4.4.3 Infiltration

After dehydration was complete, the specimens were ready for infiltration. This was done gradually to ensure satisfactory results.

The specimens were placed in a specimen bottle half-filled with pure TBA and paraplast chips were added until the solution came up to the ¾ level. The bottle was then kept on top of an oven at 60^oC to dissolve the paraplasts. After the paraplasts were completely dissolved, half of the alcohol-paraplast mixture was poured away and the volume was topped up with molten paraplasts. After 2 hours in the oven at 60^oC, the mixture was poured away completely and replaced with molten paraplasts. The molten paraplasts were changed 3 times at hourly-intervals until the materials no longer smelt of alcohol.

4.4.4 Embedding

The contents of each specimen bottle was poured into a paper tray approximately 1 x 1 x 1 cm and the specimens were arranged in a vertical or horizontal position depending on the type of material. Anthers were placed in a vertical position while ovaries and shoot apices that are difficult to place upright due to their larger size were placed horizontally. The entire mass was then cooled

until the wax block completely solidified. The wax block was stored in a dust free and clean container until required for sectioning.

4.4.5 Sectioning

The wax block was trimmed to a suitable size and shape and mounted in the specimen holder. All of the sections were cut to a thickness of 12 µm using a rotary microtome (American Optical 820) with a clean and sharp knife. Using a paintbrush, the ribbon was directed into a straight line on to stiff black paper for ease of mounting in the next step.

4.4.6 Mounting

The ribbon was cut into sections of approximately 4 cm, and transferred into warm water (40ºC) in a warming water bath and left until the sections had wholly straightened out.

A small drop of Haupt's solution (an adhesive) was placed and smeared evenly over the surface of a frosted microscope slide (76 x 26 mm) until only a barely perceptible film remained. The Haupt's solution was prepared by mixing 1 g plain gelatin, 2 g phenol crystals and 15 mL glycerin in 100 mL distilled water (Prakash, 2000). The ribbon was placed and carefully arranged on the slide in such a way to fit as many sections as possible. The mounted slide was then put on a warm plate (40ºC) for a few minutes to flatten out any wrinkles in the ribbon and to remove excess water. When the section had stretched out, the slide was taken off the warm plate, cooled down and completely dried in a low temperature oven (40ºC) for 24 hours.

4.4.7 Staining

The dried mounted-slide was placed in histolene for 30 minutes to remove the paraplast followed by the slide being transferred into a histolene-alcohol solution for 15 minutes for clearing. The slide was then transferred to 95% ethyl alcohol for 10 minutes, then passed through 80%, 70% and 50% ethyl alcohol for 5 minutes each.

The specimens were stained with 1% aqueous safranin for 24 hours, followed by two rinses in distilled water for 3 minutes each. The slide with the specimen was then passed through 50%, 70% and 90% ethyl alcohol for 2 – 4 minutes each before being stained with 0.5% fast-green in 95% alcohol for 10 seconds.

Having been stained with safranin and fast-green, the slide was washed in absolute alcohol for 10 seconds, then in a histolene-alcohol solution (1:1) for 15 seconds. It was then placed in histolene I for 15 minutes followed by placing it in histolene II for 48 hours. Finally, the slide was dried in a low temperature oven (40ºC) until completely dry.

4.4.8 Permanent mounting

A few drops of Euparal (a synthetic resin) was placed on the slide with the stained specimen. Euparal was prepared by mixing 10 mL camsal, 40 mL gum sandarac, 20 mL eucalyptol, 20 mL dioxane and 10 mL paraldehyde. This mounting medium has a refractive index of 1.48 (Prakash, 2000). A cover slip (No. 1 thickness, 50 x 24 mm) was used to cover the entire specimen. The slide was then dried in a low temperature oven (40ºC) to spread the mounting medium and dry the specimen. When completely dry, the slide was ready for microscopic examination.

4.5 Statistical analysis and data presentation

Unless otherwise stated, the experimental design used through all trials in this work was the Completely Randomised Design (CRD), either in single factor or multi factors. The results were analysed using standard Analysis of Variance (ANOVA) with the Microsoft Excel 2000 computer program (Microsoft Corporation, 2000). The Standard Error (SE) and the Fisher's Protected Least Significant Difference (FPLSD) test at $\alpha = 0.05$ was used where appropriate to identify significant differences between means (Fisher, 1966).

In addition to statistical tests, photographs were also provided. The microscopic objects were photographed using an Olympus OM-2 camera attached

to a Zeiss Standard-20 light microscope or to a Stemi SR stereo microscope for macroscopic objects. Meanwhile, normal objects were photographed using a Nikon F-601 camera with or without close-up lens attachment. All images were captured on a Kodak print film ASA 400.

The images were scanned at a resolution of 300 dots per inch (dpi) using a Hewlet Packard ScanJet 4200C flatbed scanner. The images were then manipulated using Adobe PhotoDeluxe Home Edition 3.0 software (Adobe Systems Incorporated, 1998) for clearer viewing.

CHAPTER 5
CHROMOSOME NUMBER

5.1 Introduction

Plant breeding involves an understanding of various aspects of plant genetics including the ploidy status of a given species. Therefore, in addition to its importance in systematic studies such as taxonomic revisions of a genus or species, determining the number of chromosomes of a plant is also important for genetic improvement of the plant. It is a crucial aspect and is usually the first step that should be undertaken in any plant breeding programme including the modification of ploidy levels.

The chromosome complement of a gamete is normally denoted by the letter "n" and the number of chromosomes in a single genome is represented by the letter "x". Thus, a diploid cell can be represented by the formulae of $2n = 2x$, while a haploid cell is represented by $n = x$. In addition, a tetraploid plant obtained by chromosome doubling is represented by $2n = 4x$ or $n = 2x$ in its reproductive cells. A triploid plant resulting from crossing of a tetraploid with a diploid is represented by $2n = 3x$.

Chromosome numbers have been recorded in many genera of the tribe Galegeae, sub-tribe Coluteinae (Polhill and Raven, 1981); these include *Clianthus* ($2n = 2x = 32$), *Swainsona* ($2n = 2x = 32$), *Sutherlandia* ($2n = 2x = 16$), *Lessertia* ($2n = 2x = 16$), *Colutea* ($2n = 2x = 16$), *Sphaerophysa* ($2n = 2x = 16$) and *Smirnowia* ($2n = 2x = 16$). Dawson and Beuzenberg (2000) determined *Charmichaelia nana* of the tribe Charmichaeleae as having a chromosome number of $2n = 2x = 32$. However, chromosome numbers in *Swainsona formosa* have not been recorded.

The objective of this exercise is to determine the number of chromosomes in diploid (normal) *S. formosa* as the first step in a breeding programme using *in vitro* (haploid plant induction as described in Chapter 8) or *in vivo* (tetraploid and triploid plant production as described in Chapter 10) strategies. Determining the

diploid chromosome number of untreated materials allows us to assess the success of treatments to modify the ploidy levels either via androgenesis or using an antimitotic chemical such as colchicine.

5.2 Materials and methods

5.2.1 Root tip squash

Seeds were germinated according to the procedure that was described in Chapter 4: General materials and methods. Following the 24-hour plating on moistened filter paper, the radicles started to grow from within the seeds. Roots were harvested when 1.5 cm long or 3 days after germination (Figure 9A). Root tips about 1 cm long were cut using a scalpel while holding the seed coat with forceps.

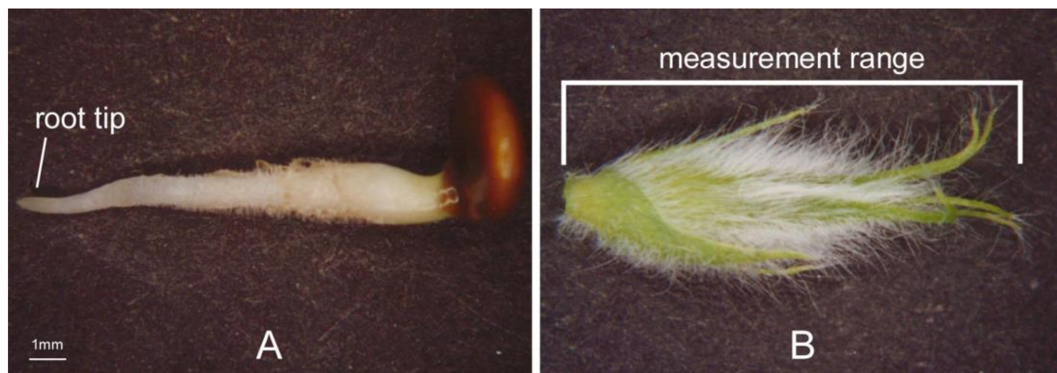

Figure 9.
Plant materials used in determining the chromosome number. A, radicle for root tip squash; B, young floral bud for anther squash.

The best time for harvesting *S. formosa* roots for the mitosis study was approximately one hour after lights were switch on; this is similar to most plant species. At this time the chromosomes at various mitotic phases can be observed clearly under a light microscope using an appropriate staining method and pre-treatment.

Harvested roots were immediately placed into a 5-mL vial half-filled with 0.1% colchicine solution for about 4 hours at room temperature. The roots were then rinsed thoroughly with distilled water and stored in a vial filled with 70%

ethyl alcohol at a low temperature (4ºC) until required. The use of colchicine as a root tip pretreatment was aimed at facilitating the chromosome counting (Prakash, 2000). Colchicine is a compound that disrupts mitosis and produces so-called arrested metaphase or prometaphase with scattered or tightly clumped chromosomes (Vaughan and Vaughn, 1998), which can be easily observed and photographed using a light microscope.

Some selected roots were taken out of the alcohol vial and rinsed thoroughly with distilled water to remove the excess alcohol. The roots were transferred into a vial containing approximately 2 mL (just enough to cover the specimens) of a stain-acid mixture (9 parts of 1% aceto-orcein + 1 part of 1 N HCl) for hydrolisation. The vial was gently warmed over an alcohol flame for about 1 minute to help the stain uptake. The roots were left in the stain-acid mixture for about 10 minutes to allow cooling down.

One root segment was taken out of the stain-acid solution and placed on a dust-free microscope slide. The root tip was cut off at approximately 0.2 – 0.4 mm from the tip; the rest of the root was discarded. Two drops of 1% aceto-orcein (without HCl) were applied to the root tip on the slide. By using a metal rod the root tip was squashed completely until a milky solution was obtained. One or two more drops of 1% aceto-orcein (without HCl) were added as necessary.

An albumin- or blood serum-coated coverslip (No. 1 thickness) was used to carefully cover the specimen. By using a piece of absorbent filter paper (Whatman No. 1) any excess stain was removed. The slide was once again warmed over an alcohol flame to remove the trapped air bubbles before being examined under a microscope.

A fast-drying nail gloss was used for sealing the edges of the coverslip to prevent the evaporation of the stain solution. By placing the slides with the sealed coverslip on a moistened filter paper in a closed Petri dish, the specimen can be stored at a low temperature (4ºC) and in a total darkness for up to one week without losing the stain quality.

A total of 60 root tips were examined so as to obtain an accurate count. These roots were harvested from germinating seeds obtained from plants that were grown over 4 seasons.

5.2.2 Anther squash

In this study, 20 plants grown in different seasons were sampled (Table 5) and young floral buds, 13.7 – 13.9 mm long (Figure 9B), were isolated. Floral buds at this size contained anthers with the majority of microspores at mother cell stage and most cells showed the mitotic metaphase. Buds were collected during the day between 10.00 a.m. and 3.00 p.m. There was no specific time for bud collection in terms of the best viewing of chromosomes. Results from another trial indicated that the size of floral buds would be an important predictor for the microspore developmental stage (see Chapter 8: Anther culture) and, therefore the examination of chromosomes within microspores and the meiosis study relied on this parameter.

The outer floral parts (sepals and petals) were removed to expose the anthers. One anther was taken out and placed on a dust-free microscope slide. Two drops of 1% aceto-orcein (without HCl) were added to the slide (Prakash, 2000). The median section of the anther was cut transversally and was gently squashed with a needle tip to release the microspores. The excess of anther material was then taken out of the stain solution and was discarded.

The stained microspores were covered with a thin coverslip and the rest of protocols were as those described for the root tip squash.

5.2.3 Image capturing

The slides were firstly examined under a light microscope (Zeiss Standard 20) with low magnification (100x) to locate the position of desired cells, followed by a higher magnification (400x). At the highest magnification (1000x), one drop of immersion oil was applied on the slides to facilitate observation.

Several cells (4 – 6) from each sample, either root tips or anthers, were counted to ensure that a consistent count was achieved. Microscopic images of

chromosomes were captured and digitised as per section 4.5 in Chapter 4: materials and methods.

5.3 Results and discussion

Examination of sampled root tips obtained from glasshouse-grown plants over several different growing seasons indicated that the number of chromosomes was uniform. Some stages of mitosis (Figure 10) were available but the chromosomes were too small for karyotype analysis. Although the pictures were not clear enough for counting the chromosomes, counting under the microscope indicated that the chromosome number was $2n = 2x = 16$. This count was confirmed by clearer pictures of the anther squash showing that $2n = 2x = 16$ in microspore mother cells (Figure 11A) and $n = x = 8$ in the pollen grains (Figure 11B).

Table 5.
Number of plants sampled for root tip or anther squashes over the 4 seasons during 2000.

Season (2000)	Root tips		Pollen grains	
	Σ plants	Σ chromosomes	Σ plants	Σ chromosomes
Spring	5	16	4	8
Summer	6	16	3	8
Winter	4	16	7	8
Autumn	5	16	6	8

Chromosome counts are known for a number of *Swainsona* species. Hair (1963) first reported chromosome numbers in *S. novae-zelandiae* as $2n = 2x = 32$. This was confirmed by Heenan (1998b) who reported the same chromosome numbers for this species now renamed *Montigena novae-zelandiae*. The same chromosome count of $2n = 2x = 32$ was also found in *S. canescens, S. occidentalis, S. cyclocarpa, S. stipularis* (Sands, 1975) and *S. galegifolia* (Ermayanti *et al.*, 1993). Following this, Buza *et al.* (2000) apparently did not make a direct count of the chromosomes of *S. recta,* but interpreted it to be an

autotetraploid based on genetic information and assumed a basic number of x = 16.

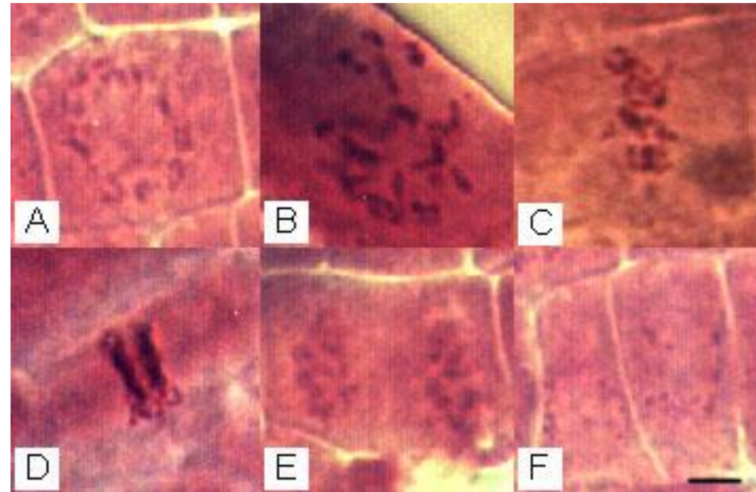

Figure 10.
Mitosis in *S. formosa* root tip cells. A, late prophase; B, late prophase-early metaphase; C, metaphase; D, early anaphase, E. late anaphase-early telophase; F, daughter cells. Bar = 12.5 µm.

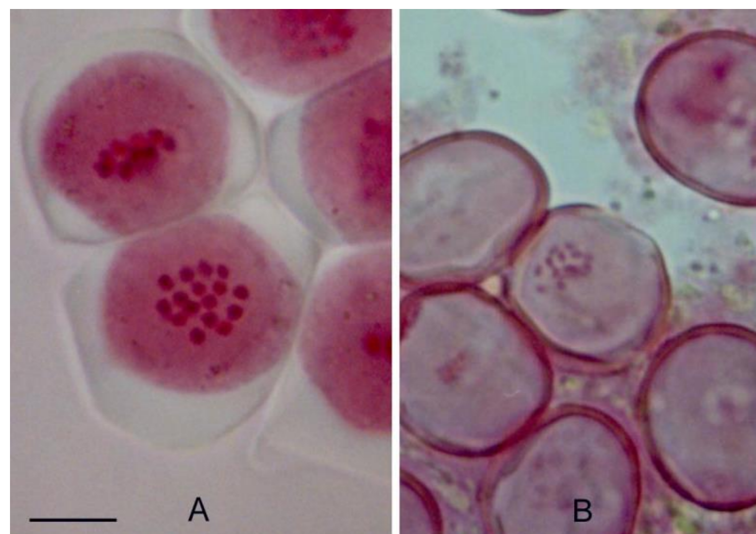

Figure 11.
Chromosomes in microspore mother cells and pollen grains of *S. formosa.* A, Microspore mother cells with 16 chromosomes ($2n = 2x = 16$); B, pollen grains with 8 chromosomes ($n = x = 8$). Bar = 10 µm.

In her revision of the genus, Thompson (1993) stated that *Swainsona* has a uniform chromosome number of $2n = 2x = 32$. Heenan (1998a) also suggested that the chromosome number of *Swainsona* species with standard modified with a boss is $2n = 2x = 32$. This differs from a group of species lacking in modifications that have a chromosome number of $2n = 2x = 16$ or 24. It seems that the chromosome number of $2n = 2x = 32$ is common in *Swainsona* and also in the Australian genus *Clianthus*. However, Sands (1975) found that the chromosome number in *S. galegifolia* was $2n = 2x = 16$. In addition, the Australian genera in sub-tribe Coluteineae such as *Sutherlandia*, *Lessertia*, *Colutea*, *Sphaerophysa* and *Smirnowia* all have species with $2n = 2x = 16$ (Polhill and Raven, 1981).

5.4 Conclusion

Based on the findings in this work it is suggested that the count of $2n = 2x = 16$ in *S. formosa* favours the basic number of $x = 8$ in *Swainsona*. This also confirms the base chromosome number of $x = 8$ in the tribe Gelegeae as previously proposed by Goldblatt (1991).

CHAPTER 6
EMBRYOLOGICAL DEVELOPMENT AND SEXUAL REPRODUCTION

6.1 Introduction

In general, plant embryology is defined as a study of the developmental process in anthers and ovules leading to the formation of seeds (Prakash, 1987). O'Connor (1991) claimed that the production of seeds was a critical factor for population maintenance. It ensures the renewal of populations and sustainability of a particular flowering plant (Rost *et al.*, 1998). However, seed production in a species is very dependent upon embryological processes starting from the formation of male and female gametes, to pollination and fertilisation until embryo formation and maturation. Any abnormality at any stage in this process will lead to the failure of sexual plant reproduction, and hence seed production.

There is considerable embryological literature on the Fabaceae to which *Swainsona formosa* belongs. However, despite many botanical and agronomic studies (Thompson, 1990, 1993; Jusaitis, 1994), there has been no adequate embryological data on this species. In fact, the importance of embryological information is not only in the evaluation of the phylogeny of taxa (Prakash, 1987; Tsou, 1997; Floyd *et al.*, 1999), but also in the development of a plant breeding programme. The anatomy of anthers and ovules, micro- and megasporogenesis, development of gametophytes, fertilisation and growth and development of the embryo, endosperm and seed coat are all important aspects in plant embryology as (Palser, 1975). Furthermore, Prakash (1987) noted that embryological data provides valuable information on many aspects of sexual reproduction, as well as on the histology of developmental sequence in anthers and ovules leading to seed formation in plants.

The objective of this work was to formulate a breeding programme for *S. formosa* by understanding its reproductive mode and studying the aspects related

to anther and ovule development, pollination and fertilisation through to seed development and germination.

6.2 Materials and methods

6.2.1 Anther, ovule and embryo development

Plant microtechnique methods as described by Prakash (2000) were used in the preparation of the materials for the study of the development of anthers and ovules of *S. formosa.* These methods consisted of fixation, dehydration, infiltration, embedding, sectioning, staining of the plant material. The stained sections were permanently mounted on slides and examined under light microscopy and photographed.

6.2.1.1 Collecting and fixing plant materials

Plant materials were obtained from plants routinely grown in a temperate glasshouse as described in Chapter 4: General materials and methods. Very young floral buds and flowers at different developmental stages until anthesis were isolated. These materials were fixed on site in FPA solution. Sepals and petals, where possible, were removed prior to fixing.

6.2.1.2 Embedding and sectioning

Very young floral buds were processed as a whole, while the pistil and anthers of older flowers were removed, and petals and sepals were discarded. The specimens were treated with the procedure outlined in Chapter 4, i.e. dehydrated in a series of TBA, infiltrated and embedded in paraplast, and finally sectioned at 12 μm thickness. The paraplast ribbons were mounted on microscope slides and kept overnight in a 40°C oven to dry.

6.2.1.3 Staining

Double staining with safranin-fast green was used to stain the specimens. Dry sections mounted on the slides were dewaxed in Histoclear and dehydrated through an ethanol series of descending concentrations. Following this, the slides

were soaked in 1% safranin "O" for 2 hours, rinsed in tap water followed by distilled water, dehydrated through an ascending ethanol series, and stained in 0.5% fast-green in 95% ethanol for 15 seconds. The slides were transferred to an absolute alcohol solution for a few seconds to remove the excess stain, cleared in Histoclear solution for the next 24 hours, and finally mounted in Euparal.

6.2.1.4 Image capturing

The specimens were examined under light microscopy and the images were captured and digitised as per section 4.5 in Chapter 4: Materials and methods.

6.2.2 Pollen germination

To study the pollen germination, flowers on the second day of anthesis were selected from 5 different plants grown in the glasshouse. Dry mature pollen grains from each individual flower from 3 – 4 umbels were collected in a small glass vial and mixed before being cultured *in vitro*.

6.2.2.1 Pollen viability and rate of pollen tube formation

A preliminary trial was conducted to determine the pollen viability and rate of pollen tube formation at different times, i.e. 5-10, 11-15, 16-20, 21-25 and 26-30 minutes after germination. Pollen germination was undertaken on BK medium (Brewbaker and Kwack, 1963) containing 720 ppm $Ca(NO_3)_2 \cdot 4H_2O$, 200 ppm $MgSO_4 \cdot 7H2O$, 200 ppm KNO_3, 20 ppm H_3BO_3 supplemented with 10% sucrose. Using a paintbrush, pollen was dusted onto and spread evenly over the surface of one drop of this medium on a microscope slide. The slides were placed in closed Petri dishes on a layer of moistened filter paper. The dishes were kept at room temperature to allow the pollen to germinate. Five replicates for each treatment were used and the means of pollen tube formation were derived from 10 fields of view for each replicate. The observations were undertaken with a light microscope at a magnification of 400x. The results from this trial were used in the assessment of pollen germination in the next trial.

6.2.2.2 The effect of sucrose on pollen tube formation

In the second trial pollen grains were germinated on the same medium with different concentrations of sucrose, i.e. 0, 5, 10, 15 and 20%. Based on the results of the previous trial, pollen tube formation was assessed with a light microscope, 30 after the pollen had been spread on the medium.

The trial was arranged in a completely randomised design with 5 replicates. Each replicate consisted of 10 fields of view. The percentage of germinated pollen was determined by counting the number of pollen grains forming tubes divided by all pollen grains seen in one field of view at a magnification of 400x. An ANOVA followed by the FPLSD test (Fisher, 1966) at $\alpha = 0.05$ were used in data analysis. The Standard Errors of means were also calculated.

For both experiments only pollen grains with tube formation at least half the diameter of the pollen grain were counted.

6.2.3 Seed germination

Seed germination was studied using 6-month old seeds stored at 4°C. Three temperature regimes were tested, i.e. 20, 25 and 30°C. Following scarification with just-boiled water for 10 seconds, seeds were germinated on a double-layered filter paper in Petri dishes. To study the type of germination, scarified seeds were also germinated in Jiffy pots, and the pots were kept under an intermittent moist spray in a shaded glasshouse.

Each treatment consisted of 5 replicates containing 20 seeds each, and the 5 replicates were arranged in a completely randomised design. An ANOVA was used in data analysis. The FPLSD test (Fisher, 1966) at $\alpha = 0.05$ was employed to separate the means and the Standard Errors of means were also calculated.

6.3 Results

6.3.1 Floral parts

In young floral buds, all sexual organs and the petals were enclosed tightly within the sepals (Figure 12A). As the flowers developed, the red-coloured petals elongated rapidly beyond the green sepals resulting in a combination of red-green

floral buds. Five sepals and five petals were present within the flowers. Five to seven flowers growing at the end of each peduncle collectively formed the umbel or inflorescence.

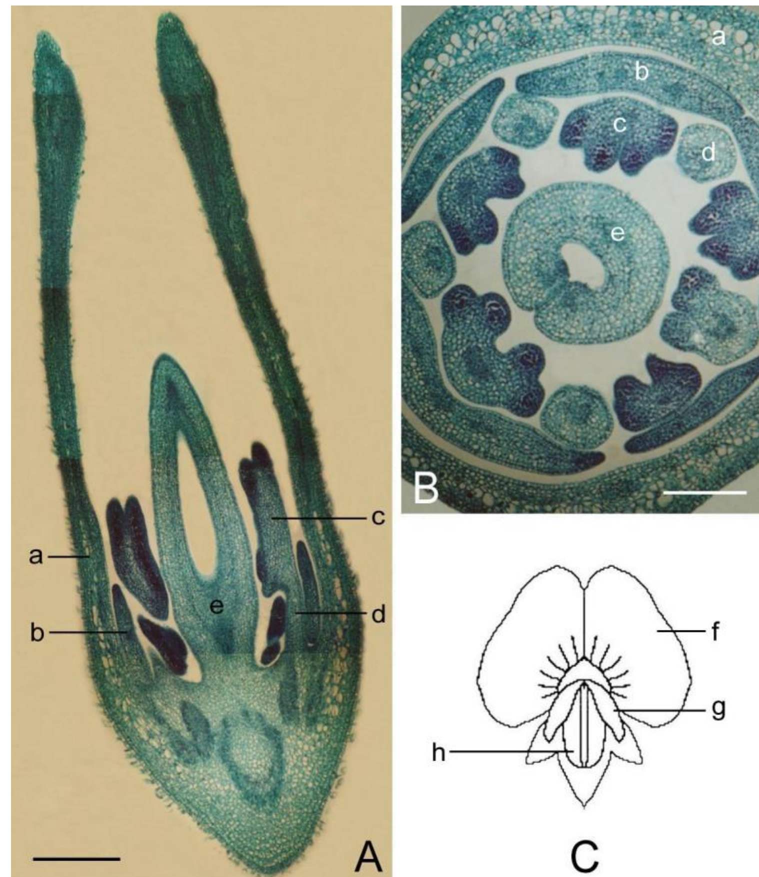

Figure 12.
Sectioned floral bud of *S. formosa* showing internal structures (A and B) and schematic drawing of a typical legume flower (C). (a, sepal; b, petal; c, anther; d, filament; e, pistil; f, standard; g, wing; h, keel). Bar = 100 µm.

There were ten anthers in each flower. The filaments of nine anthers were fused and elevated as a tube whereas the posterior anther was separated from the other nine filaments (Figure 13). Young anthers were semi-transparent and green while the mature anthers were yellow (Figure 14). Anther dehiscence took place before the flower opened, but anther dehiscence and flower opening occurred on the same day. It was found that anthesis took place mostly between 8.00 and 10.00 a.m. and flowers were fully opened at around 3.00 - 4.00 pm. The stigma

has also been found to be receptive one day before the dehiscence of the anthers (Chapter 7: Pollen longevity and stigma receptivity).

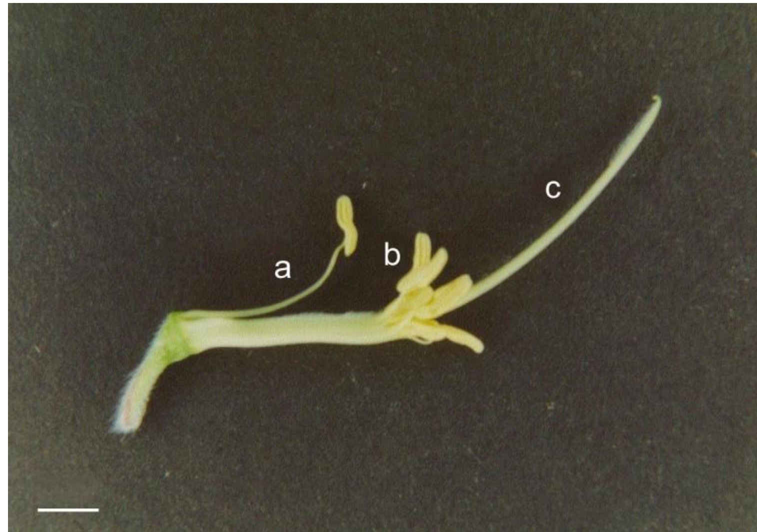

Figure 13.
Sexual structures of the flower of *S. formosa*. (a, posterior stamen remains separate from the other nine stamens; b, the filaments of nine stamens fused into a tube; c, pistil). Bar = 5 mm.

Figure 14.
External appearance of anthers of *S. formosa*. A, young anther; B, mature anther. Bar = 1 mm.

The pistil was unicarpelate and covered by anterior-facing trichomes (Figure 15). These trichomes have a key role in pollen extrusion from the keel during bird pollination (Arroyo, 1981) and also restrict self-pollen access to the stigma (Jusaitis, 1994). As the flower developed, the pistil elongated beyond the anthers and curved back toward the free anther. As in most papilionoid legumes, a layer of stigmatic cuticle (membrane) was found to cover the stigma of *S. formosa.* The stigmatic cuticle needed to be ruptured to allow pollen germination on the stigma. Lord and Heslop-Harrison (1984) suggested that such rupture could be achieved by tripping the flower, by mechanical disturbance or increasing fluid pressure in the style.

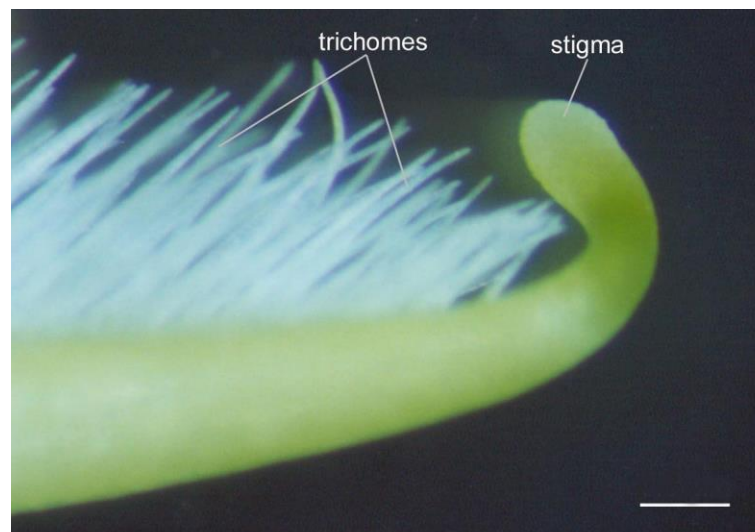

Figure 15.
The pistil of *S. formosa*, covered by trichomes, elongated and curved back toward the posterior anther. Bar = 0.5 mm.

6.3.2 Anther and ovule development

6.3.2.1 Anther anatomy

Microscopic observation showed that the anthers of *S. formosa* consisted of four compartments or locules, i.e. the anthers are tetrasporangiate (Figure 16A-F). In the early stage of anther wall development, the hypodermal cells divide periclinally to form the primary parietal and primary sporogenous cells. The sporogenous cells divide and differentiate into microspore mother cells (microsporocytes). Meanwhile, the primary parietal cells divide periclinally to

form the outer and inner secondary parietal cells. The outer secondary parietal cells underwent further division resulting in the endothecium and one middle layer. The inner secondary parietal cells develope into the secretory tapetum, the food-rich layer of cells (Figure 17A-C).

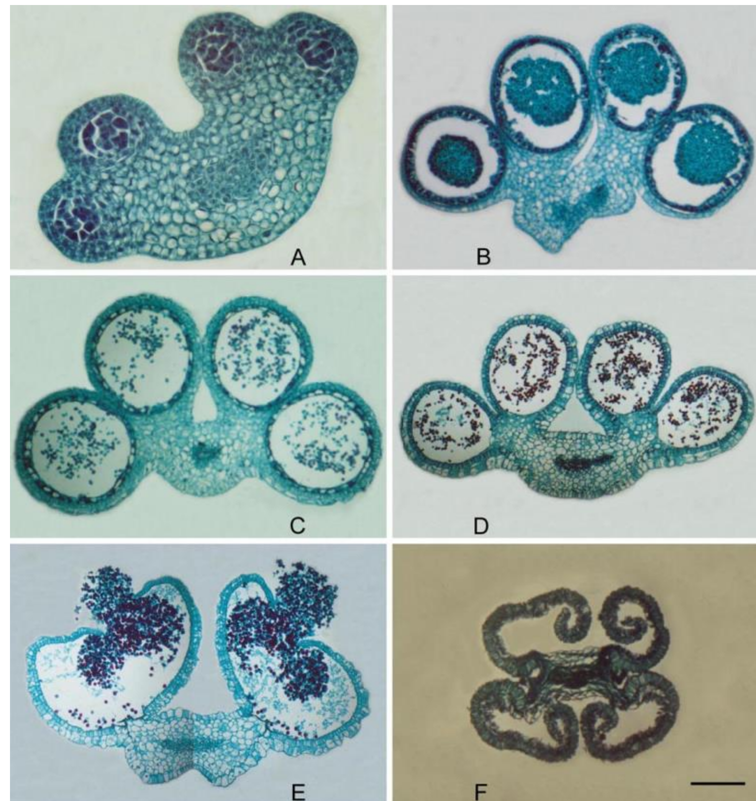

Figure 16.
Cross section of anther of *S. formosa* at different stages. A, anther containing microspore mother cells; B, anther containing mother cells undergoing meiosis; C, anther containing young pollen grains; D, anther containing mature pollen grains; E, anther at dehiscence stage; F, dehisced anther. Bar = 100 µm.

The mature anther wall in *S. formosa*, which lay under the single-layered epidermis, consisted of three layers, one each of endothecium, middle layer and tapetum (Figure 18B). The epidermis, which did not develop from primary parietal cells (Prakash, 1987) and so is not morphologically part of the anther wall, was present throughout anther development. However, the middle layer and tapetum degenerated before or during meiosis leaving only the endothecium and the epidermis (Figure 18C). The endothecium developed fibrous thickening on

the radial and inner tangential walls when microspore development was at the uninucleate stage (Figure 18D), and enabled the mature anthers to dehisce and the pollen to be dispersed.

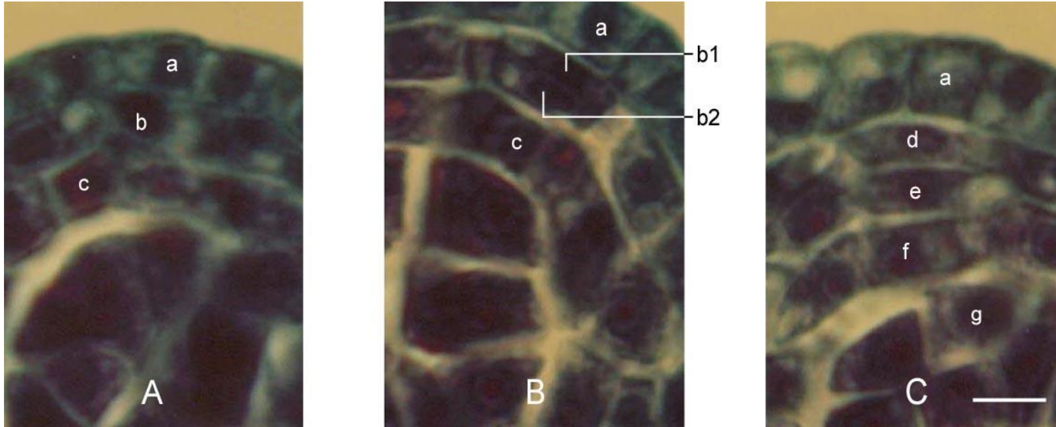

Figure 17A – C.
Stages in anther wall development in *S. formosa*. (a, epidermis; b, outer secondary parietal cell; b1, outer secondary parietal cell developing into endothecium; b2, outer secondary parietal cell developing into middle layer; c, inner secondary parietal cell developing from hypodermal cell; d, endothecium; e, middle layer; f, tapetum; g, sporogenous cell). Bar = 10 µm.

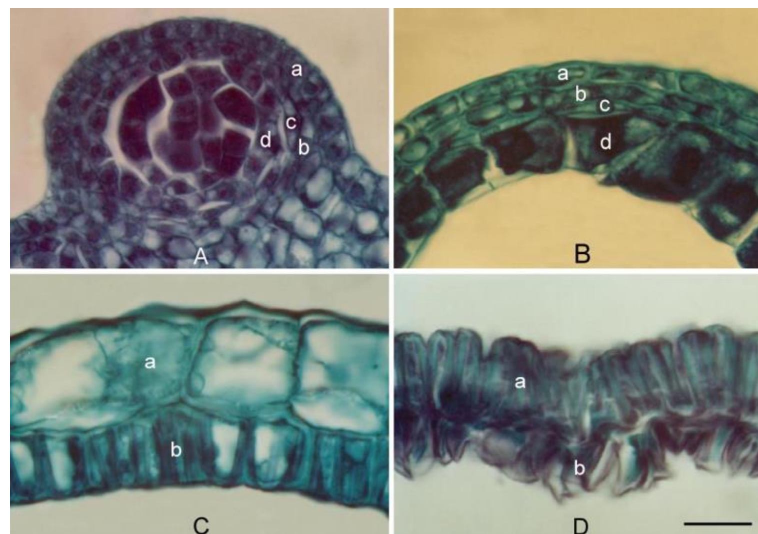

Figure 18.
The differentiation of the anther wall in *S. formosa.* A, wall of young anther at mother cell stage; B, wall of anther just before meiosis; C, anther wall after meiosis; D, anther wall after dehiscence. (a, epidermis; b, endothecium; c, middle layer; d, tapetum). Bar A = 25 µm, B-D = 10 µm.

Anther dehiscence started from the broken septum, which separated the two sporangia on each side. This broken wall resulted in an opening through which the pollen grains were released (Figure 19A,B).

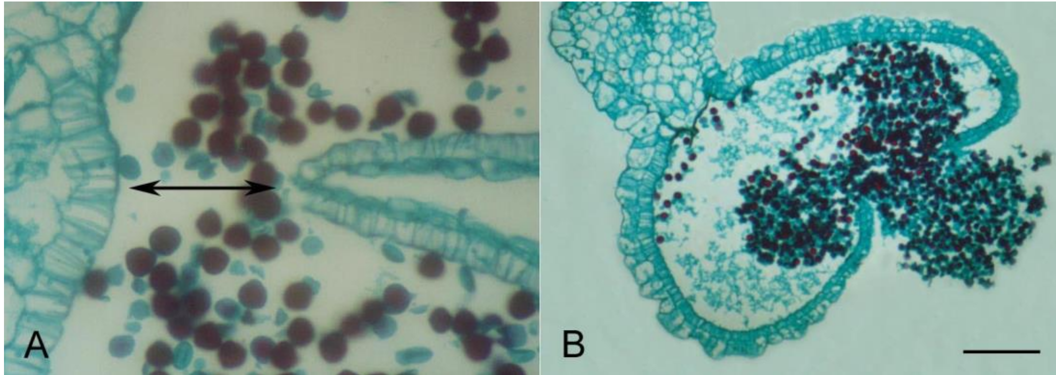

Figure 19.
Stages in anther dehiscence in *S. formosa*. A, broken septum as indicated by arrowheads; B, pollen grains are released from within the anther. Bar A = 25 µm, B = 100 µm.

6.3.2.2 Microsporogenesis

There were abundant microspore mother cells in each sporangium giving rise to a large number of pollen grains which has been a major problem in the commercialisation of *S. formosa* as cut flowers.

At the initiation of meiosis, the microspore mother cells were surrounded by callose (Figure 20A-G), which persisted until the formation of microspores. There was a simultaneous cytokinesis of microspore mother cells following meiosis resulting in the tetrahedral shape of the microspore tetrads (Figure 20G) that were still enclosed in callose. A strong correlation was found between the microspore developmental stage and floral bud length (Chapter 8: Anther culture).

Furrowing was found to accompany the formation of the microspore wall (Figure 21A). The microspores were initially isolated from one another by the presence of callose. As the callose disappeared due to dissolution, individual microspores were released into the sporangium (Figure 20H), by which time a thin exine layer and incipient pores became evident on the wall. The exine layer underwent thickening as the microspore developed into early-binucleate stage

(Figure 21B). Furthermore, mitosis took place and the microspore grew into a 2-celled pollen grain (Figure 21C). By the time the microspores became mature pollen grains, the tapetum and middle layer were broken down leaving only the fibrous endothecium and the epidermis (Figure 18D). Three pores were found on the mature pollen grains, and therefore they were classified as triporate (Tootil, 1984).

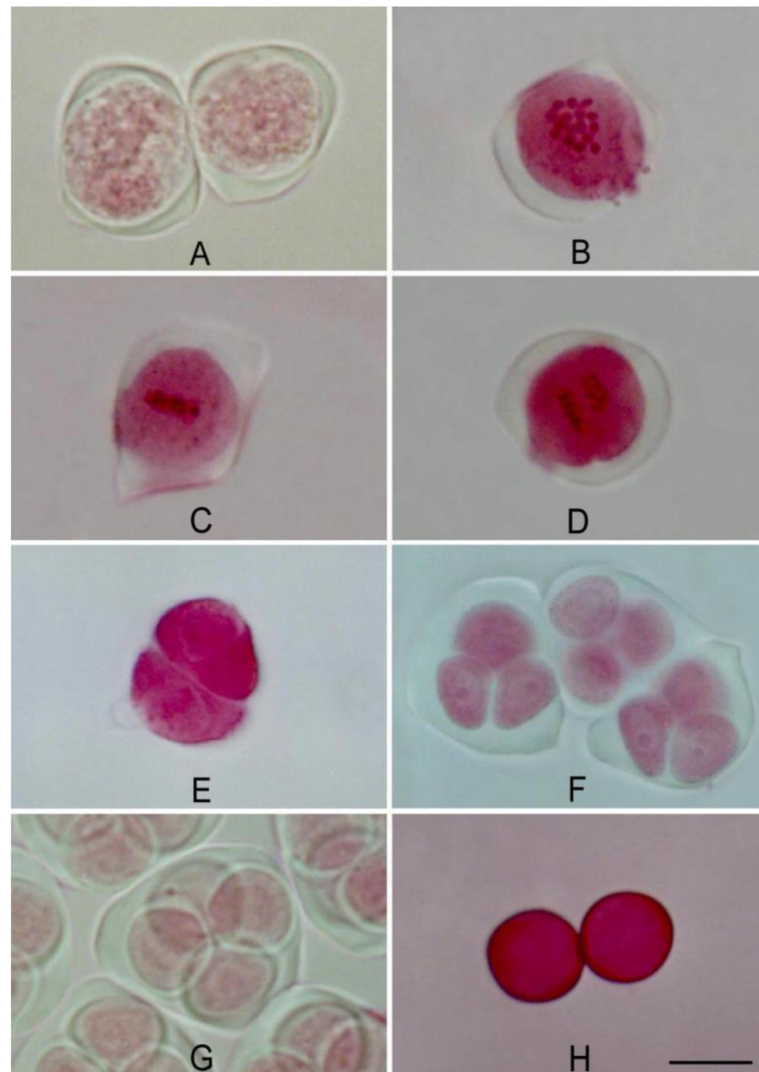

Figure 20.
Microsporogenesis leading to the formation of tetrads in a tetrahedral arrangement in *S. formosa*. A, mother cells before meiosis; B-E, mother cells undergoing meiosis; F-G, tetrads of microspores embedded in the callose wall; F, early-uninucleate pollen grains. Bar = 10 µm.

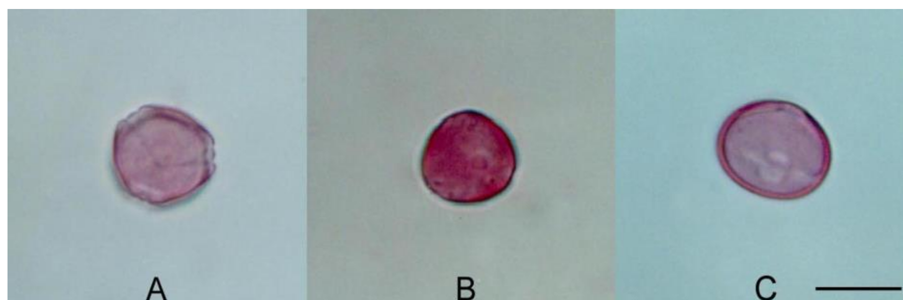

Figure 21.
Differentiation of mature pollen grain in *S. formosa*. A, pollen with thin exine layer and three incipient pores; B, pollen at early-binucleate stage; C, mature pollen showing thick exine layer. Bar = 10 µm.

6.3.2.3 Pollen germination

The result of *in vitro* germination attempted on BK medium (Brewbaker and Kwack, 1963) containing 10% sucrose is presented on Figure 22. Pollen tubes started to grow within the first 10 minutes of culture (28.3% pollen tube formation) but the length of most pollen tubes was less than half of the respective pollen diameter. Tube formation increased dramatically within the next 10 minutes (48.84%) and remained steady after 60 and 120 minutes, after the pollen was initially spread (63.70 and 63.71% pollen germination, respectively). Based on these results, pollen germination on the next trial was assessed at 120 minutes after culture initiation.

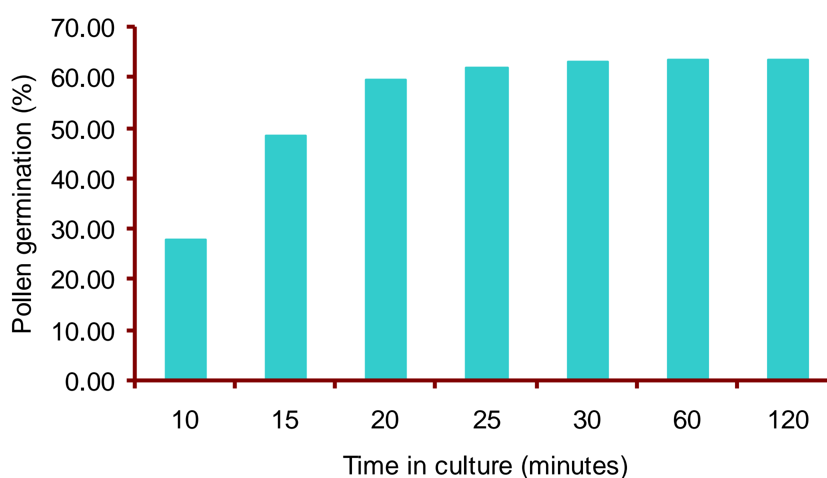

Figure 22.
The percentage of germination of *S. formosa* pollen at various times after culture initiation.

Analysis of variance on the effect of sucrose on *in vitro* pollen germination showed that the number of germinating pollen grains was highly dependent on the presence of sucrose in the media ($P < 0.01$). Further analysis using FPLSD indicated that the medium with 10% sucrose produced the highest percentage of pollen germination (64.14%). However, it was not significantly different from the 15% sucrose medium, which produced 62.91% pollen germination. A further increase in sucrose concentration up to 20% resulted in a significant reduction in germination (48.92%). Significantly lower percentages of pollen germination (19.64 and 43.58%) were also found with sucrose concentrations of 0 and 5%, respectively.

The effect of sucrose concentration on the germination of pollen grains of *S. formosa* is presented on Figure 23.

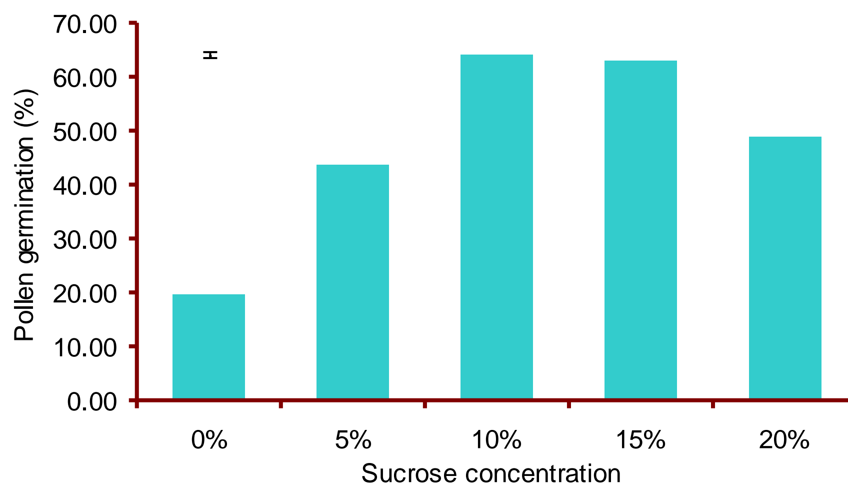

Figure 23.
The effect of sucrose concentration on pollen tube formation in *S. formosa*. Bar represents SE ± 0.51.

With sucrose concentrations of 10 – 15% pollen tubes started to grow within 5 – 10 minutes but the length of the tubes was less than a half the pollen diameter (Figure 24B). Within the next 30 minutes the rate of tube growth increased rapidly and most pollen produced a tube length approximately 1.5 times the pollen diameter. About sixty minutes after germination most pollen tubes reached a length of 3 – 4 times the pollen diameter (Figure 24C).

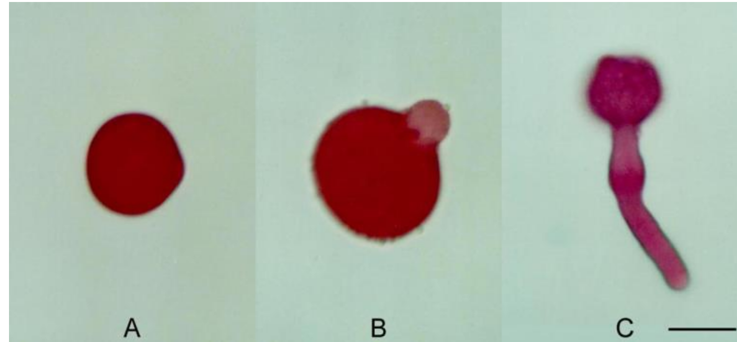

Figure 24.
The growth of pollen tube from culture initiation to 60 minutes after germination on BK medium supplemented with 10% sucrose. A, pollen grain at the time of culture initiation; B, ten minutes after germination; C, sixty minutes after germination. Bar A and B = 10 µm, C = 25 µm.

6.3.2.4 Ovule

The ovary of *S. formosa* was round in shape with two locules (ovarian cavity). Each locule consisted of one row of up to 30 ovules. The ovule developed horizontally and the funiculus appeared to be attached half way between the chalaza and the micropyle, forming the campylotropous orientation (Figure 25). The ovules in the ovary developed simultaneously but resulted in non-uniform maturity. Ovules at the apical region of the ovary reached maturity faster than ovules at the basal region.

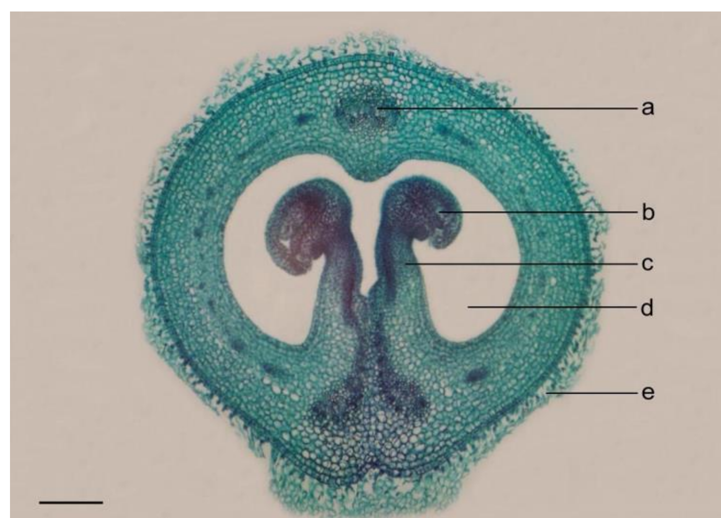

Figure 25.
Transverse section of ovary of *S. formosa*. (a, vascular bundle; b, ovule; c, funicle; d, locule; e, trichomes). Bar = 0.1 mm.

A single archesporium was present in the ovule. This archesporium originated from one of the hypodermal cells, which divided in a periclinal direction to form a primary parietal cell and a sporogenous cell. The sporogenous cell differentiated into a megasporocyte and the primary parietal cell developed into 2 nucellar layers above the megasporocyte (Figure 26).

The ovular primordium was dome-shaped and initiated from the marginal placenta as homogenous hemispherical protuberances (Figure 27A-F). The primordia started to undergo curvature before the differentiation of integuments (Figure 27F).

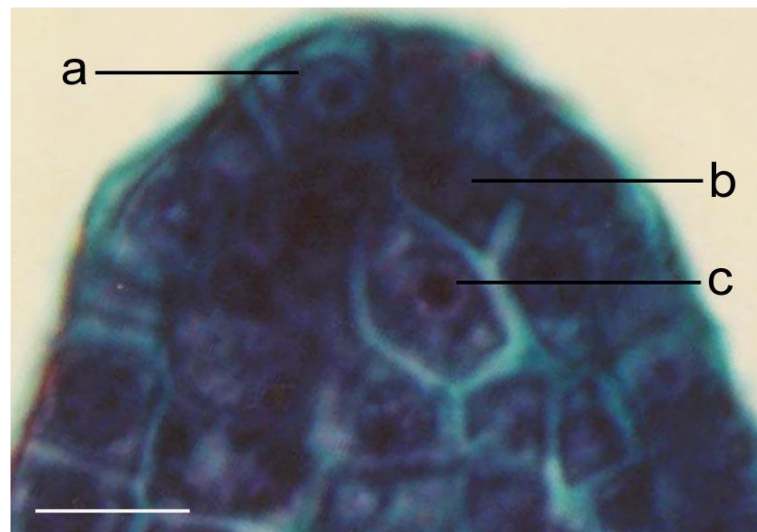

Figure 26.
Young ovule of *S. formosa* showing sporogenous cell. (a, nucellar epidermis; b, parietal cell; c, sporogenous cell). Bar = 10 µm.

The outer and inner integuments developed simultaneously from the nucellus at the sporogenous cell stage. Initially, both outer and inner integuments developed in a synchronous manner (Figure 28A,B) but later the outer integument developed more rapidly than the inner integument (Figure 28C) resulting in the enclosure of the inner integument and the nucellus at the megasporocyte stage. Both outer and inner integuments at this stage consisted of two layers of cells. The outer integument continued to grow and completely envelope the nucellus at the completion of megasporogenesis (Figure 28D), whereas the inner integument

reached only the tip of nucellus (Figure 29A). By the 2-nucleate stage of the female gametophyte the inner integument reached the micropyle while cells of the outer integument continued to divide anticlinally and periclinally in the micropylar region resulting in a collar 6 – 8 cells thick at the tip (Figure 29B).

The cells in the outer integument continued to divide repeatedly as the ovule grew to maturity. This repeated division resulted in a massive structure of cells growing towards the funiculus and covering the inner integument and the micropylar end of the nucellus. This resulted in a zigzag micropyle (Figure 29B). Accumulation of starch took place in the outer and inner integuments in the vicinity of micropyle.

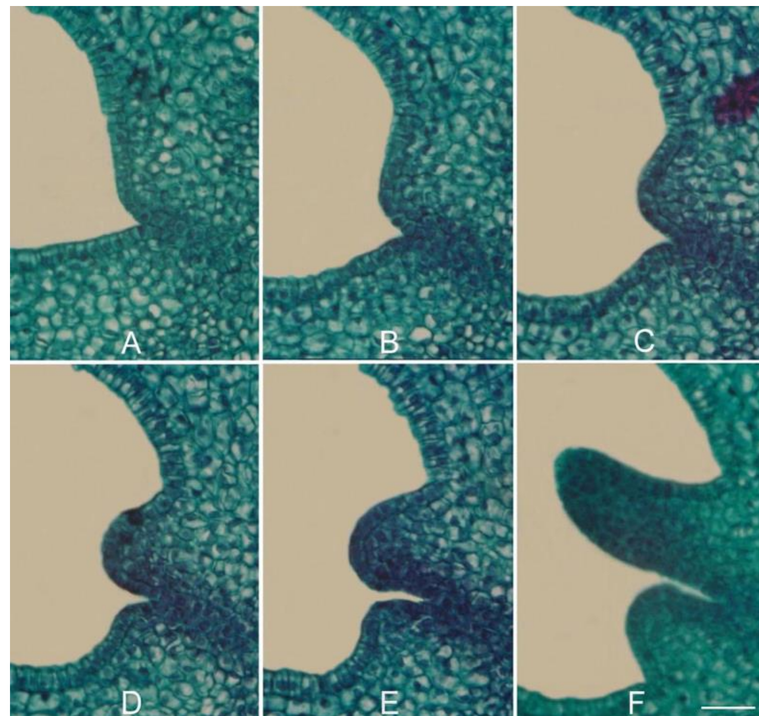

Figure 27A-F.
The initiation of ovule development, in *S. formosa*, from the marginal placenta.
Bar = 10 µm.

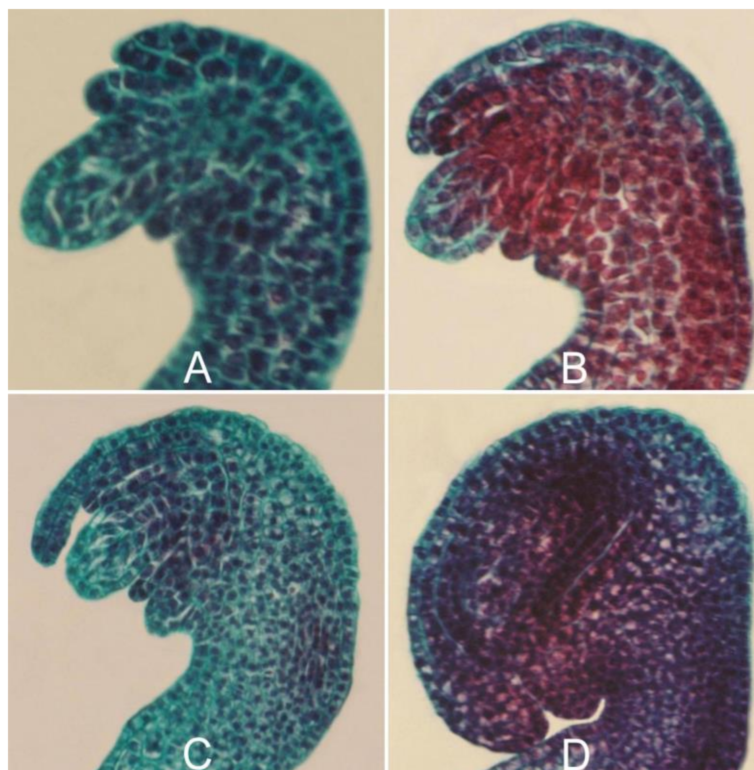

Figure 28A-C.
Stages in the development of the outer and inner integuments of the ovule of *S. formosa*.

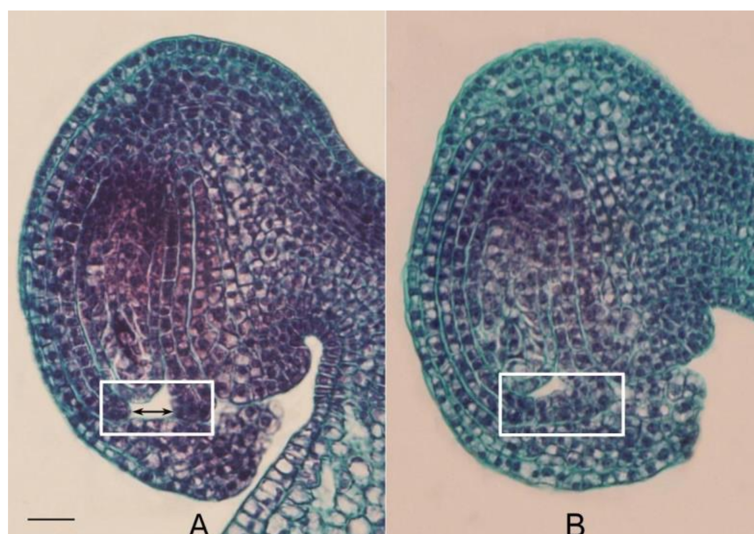

Figure 29.
Final development of the integuments in *S. formosa* (boxed areas). A, outer integument completely enveloping the nucellus at the end of megasporogenesis; B, inner integument reaches micropyle by the 2-nucleate stage. Bar = 10 µm.

The female gametophyte increased in size at the expense of nucellar tissue, particularly at the sides and in the micropylar region. As a consequence of this, the inner integument on the sides, the single-layered nucellar epidermis at the micropylar end and the hypostase in the chalazal region border the mature female gametophyte.

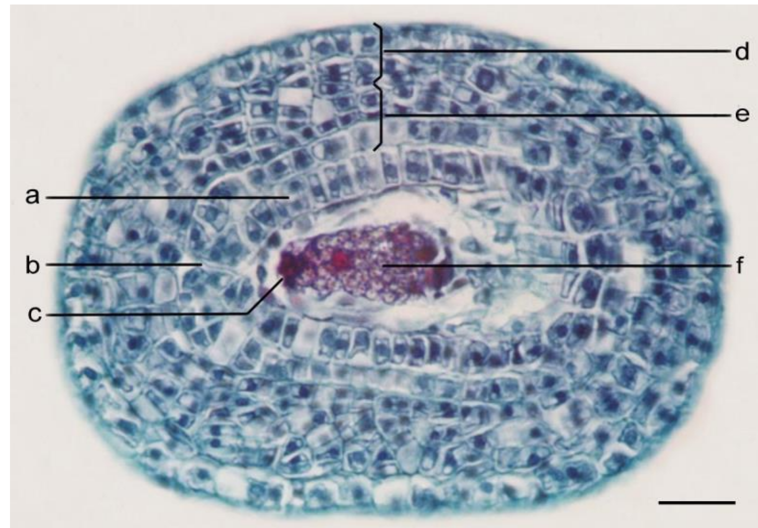

Figure 30.
Starch accumulation in the inner side of nucellar epidermis of *S. formosa*. (a, nucellar epidermis; b, micropyle; c, embryo sac; d, outer integument; e, inner integument; f, starch grains). Bar = 25 µm.

During the development of the female gametophyte, starch grains were deposited in the nucellar epidermis (Figure 30). The presence of starch was abundant in the outer and inner integument as well as in the mature female gametophyte.

An interesting finding in the ovule of *S. formosa* was the presence of more than one embryo sac (Figure 31), however, of a total of approximately 1820 seeds germinated throughout the experiments, only one (0.06%) twin seedling was found (Figure 32A). This is interesting because the occurrence of multiple embryos and germination of twin seedlings are uncommon in Fabaceae. The very low incidence of twin seedlings suggested that only one mature embryo is normally formed in the seeds of *S. formosa.*

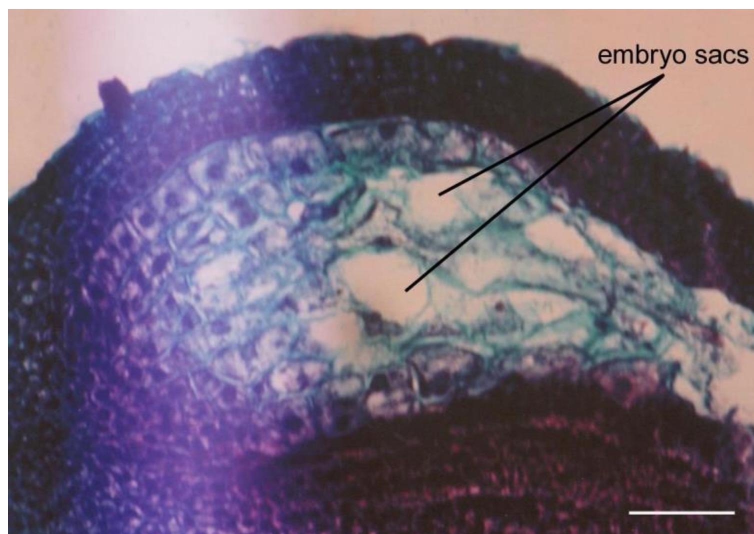

Figure 31.
The occurrence of multiple embryo sacs in *S. formosa*. Bar = 25 µm.

The megaspore mother cell underwent meiosis leading to the formation of a linear tetrad of megaspores. Of these four cells only the one in the chalazal region functioned, whereas the three micropylar ones degenerated. The functional megaspore underwent successive karyokinesis resulting in 2-nucleate, then 4-nucleate and finally 8-nucleate female gametophytes. In the mature embryo sac, two synergids, two polar nuclei and three antipodal cells were found (Figure 33). The polar nuclei lay towards the centre of the embryo sac and were surrounded by starch grains.

Figure 32.
The comparison of twin and normal seedling of *S. formosa*. A, twin seedling; B, normal seedling.

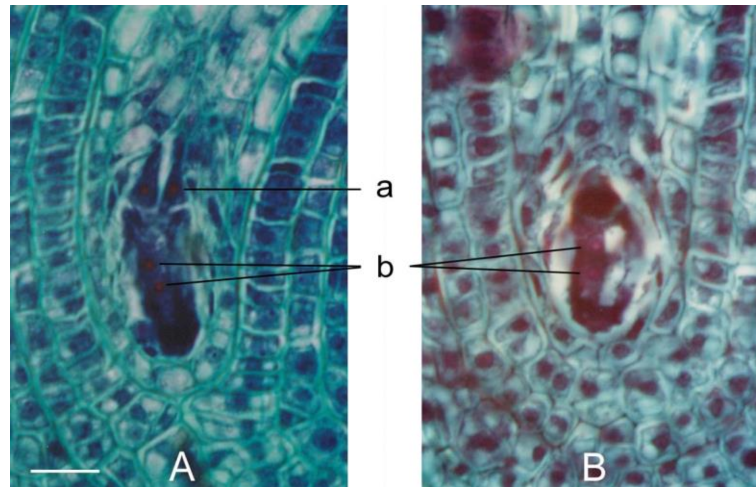

Figure 33.
Mature embryo sac of *S. formosa*. A, longitudinal section; B, oblique section. (a, antipodal cells; b, polar nuclei). Bar = 10 µm.

6.3.2.5 Pollination and fertilisation

S. formosa is a self-compatible plant, however, as with most papilionoid legumes (Shivanna and Owens, 1989) pollination is often hampered by the presence of a stigmatic cuticle that prevents pollen germination until the stigmatic cuticle is ruptured. In this study, pollination was enhanced by rubbing the receptive stigma with a fingertip covered with pollen grains from the same flower. This method of hand-pollination enhanced pollen germination on the stigma (Figure 34) and resulted in 100% pod formation. Jusaitis (1994) reported that rubbing the stigma was found to be a more effective way for successful pollination in *S. formosa* compared to just touching the undisturbed stigma with pollen. In natural conditions, pollination in *S. formosa* may involve New Holland honeyeaters (*Phylidoniris novaehollandiae*) which feed on the nectar (Jusaitis, 1994).

Upon germination, the pollen tubes travelled down the stylar canal and grew along the placenta towards the nucellus through the micropyle. The pollen tube entered the embryo sac via one of the synergids, destroying the synergid in the process. The other synergid degenerated soon after fertilisation occurred.

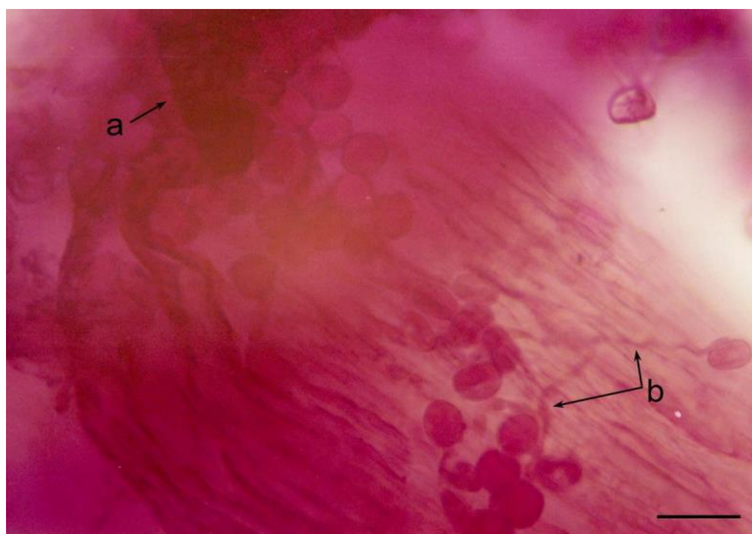

Figure 34.
Pollen germination on the stigma of *S. formosa*. (a, peel-off stigmatic cuticle; b, pollen tubes). Bar = 25 µm.

6.3.2.6 Endosperm and embryo

As with most members of the Fabaceae (Prakash, 1987), the development of the endosperm in *S. formosa* was nuclear. The primary endosperm nucleus divided following the enlargement of the embryo sac and the accumulation of starch grains. Large starch grains accumulated particularly in the close vicinity of the micropyle. As the zygote continued to grow, starch accumulation proceeded gradually from the micropylar region to the chalazal end.

The zygote enlarged and underwent successive divisions resulting in an early globular embryo (Figure 35). Mature seeds had only one or two layers of endosperm (Figure 36) as the embryo consumed the endosperm during its development. The mature embryo had two equal cotyledons. The embryo in the mature seed consumed almost all the endosperm, and the reserve food was transferred to the cotyledons. The period of seed development from a successful pollination to harvesting was about 60 days.

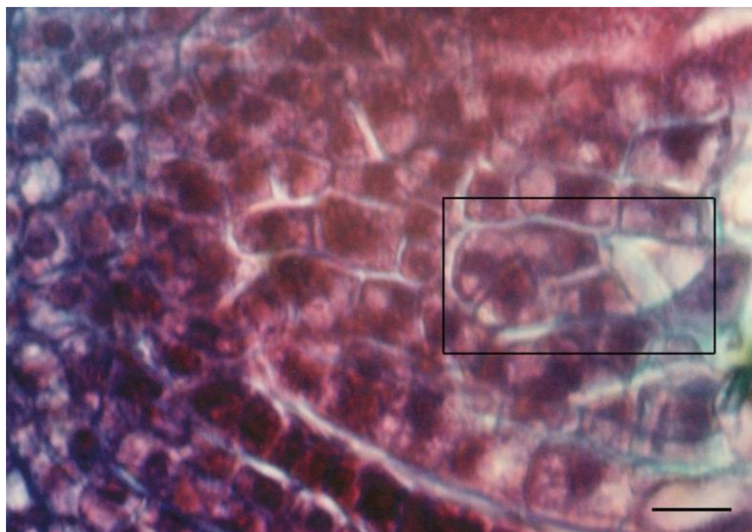

Figure 35.
Early-globular embryo stage (box) of *S. formosa.* Bar = 10 µm.

6.3.2.7 Seed coat

The female gametophyte underwent enlargement after fertilisation resulting in seed formation. Until the mature embryo sac stage the outer and inner integuments consisted of two layers of cells. The cells of the inner integument divided anticlinally and underwent radial elongation becoming 2- or 3-layered until these cells disappeared at the globular embryo stage.

Meanwhile, the outer epidermal cells of the outer integument also divided anticlinally and differentiated into radially elongated cells. The layer beneath this did not show any differentiation. The two cell layers of the outer integument enlarged and acquired wall thickenings during food reserve deposition in the cotyledons. By the time the cotyledons formed, the outer integument had increased to approximately 8 – 10 layers of cells (Figure 37). In mature seeds, the outer layer of the outer integument consisted of thick-walled palisade cells. A layer comprising osteosclereids that are called “hour-glass” cells (Figure 38) were present in the layer beneath the palisade cells. The remaining integumentary tissues, mainly thin-walled parenchyma, were crushed as the seeds matured.

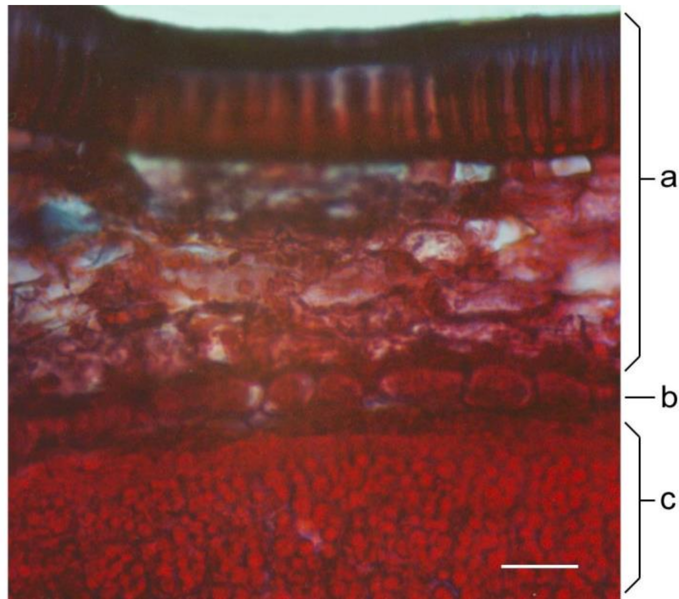

Figure 36.
Transverse section of seed of *S. formosa*. (a, seed coat; b, one layer of endosperm; c, part of cotyledon part). Bar = 10 µm.

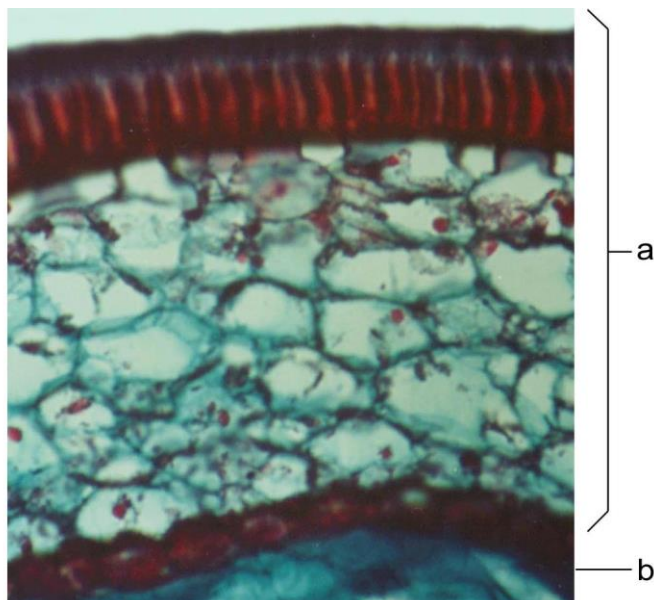

Figure 37.
Transverse section of seed coat of *S. formosa* showing an increased number of cell layers of the outer integument. (a, outer integument; b, nucellus).

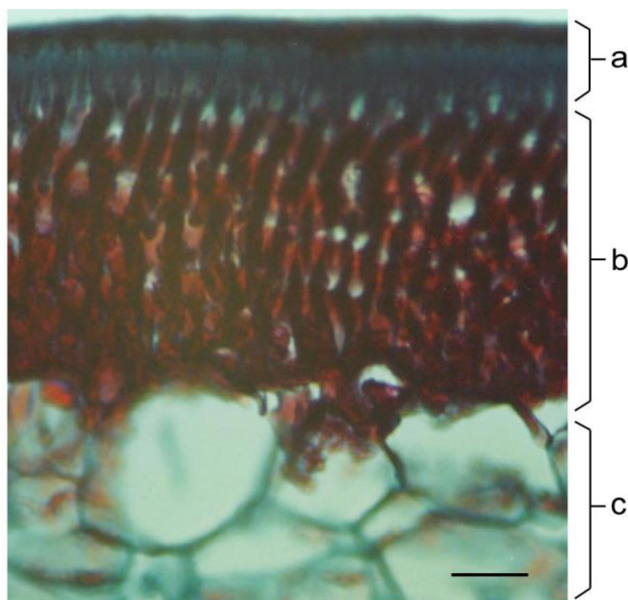

Figure 38.
Structure of the outer integument showing the differentiation of the layers. (a, palisade cells; b, osteosclereids; c, undifferentiated layer. Bar = 10 µm.

6.3.2.8 Seed germination

Seed germination was significantly affected by temperature ($P \leq 0.01$). Among the three temperature regimes tested, the temperature of 25°C showed the best effect on seed germination as indicated by the highest germination rate (97%) on the second day. This was significantly different to the temperatures of 20°C or 30°C, which produced 62.5% and 40% seed germination, respectively (Figure 39). However, seeds that were germinated at 25°C were found to have the shortest radicles (6.88 mm) compared to the other two temperature regimes. The longest radicle (10.45 mm) was recorded on seeds germinated at 30°C followed by 8.02 mm on seeds germinated at 20°C.

Seed germination attempted in the Jiffy pots showed an epigeal type of germination (Figure 40). The radicle started to grow on the second day of sowing. On the fourth day the hypocotyl arch straightened and lifted the cotyledons still wholly covered by the testa. As the cotyledons expanded, the seed coat was pushed forward. On the fifth day the cotyledons separated from each other and the seed coat dropped to the ground.

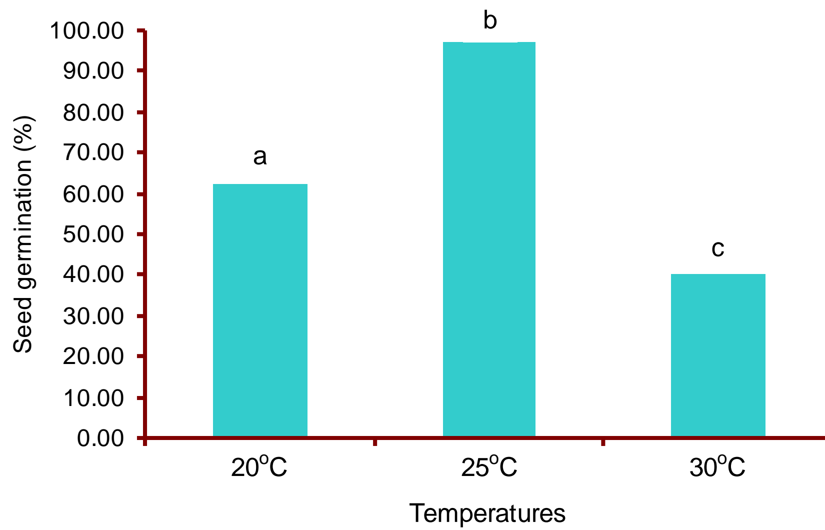

Figure 39.
The effect of environmental temperature on the percentage of seed germination in *S. formosa*. Mean separation was based on FPLSD at $\alpha = 0.05$.

The first leaves appeared on the 10th day after germination, but the spear-shaped leaflet that is characteristic of mature plants did not appear until 3 weeks of germination. Unlike the cotyledons of *Glycine max* that were shed on the 13th day after germination (Prakash and Chan, 1976), the cotyledons of *S. formosa* remained on the plants until flowering time.

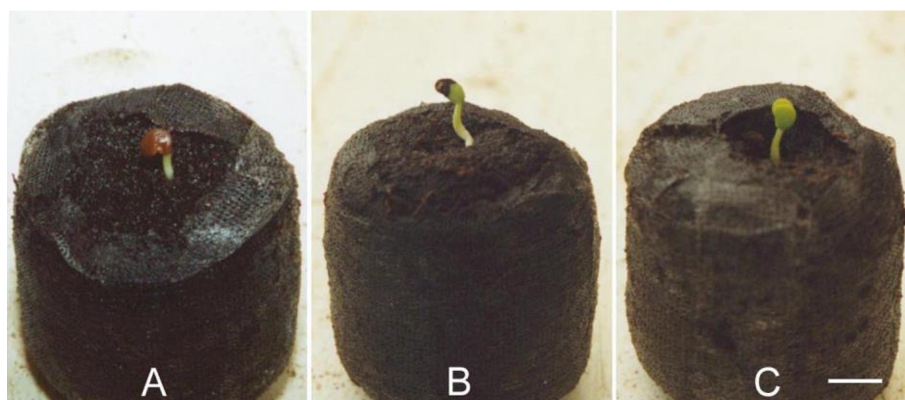

Figure 40.
Epigeal type of seed germination in *S. formosa*. A, emerged seedling with cotyledon still covered with testa; B, cotyledon more visible and testa being shed; testa totally dropped. Bar = 1 cm.

6.4 Discussion

The floral characteristics of *S. formosa* are those typical of a legume flower. Anther dehiscence occurred before the flower was opened and the stigma was receptive one day before anther dehiscence. This supported the reports of previous authors (Williams, 1996; Kirby, 1996a, 1996b) that *S. formosa* is fully self-pollinated. However, self-pollination is often hampered by the presence of the stigmatic cuticle (Jusaitis, 1994) that prevents pollen grain germination until the stigmatic cuticle is ruptured. The development of the stigma is similar to that reported in *Phaseolus vulgaris* (Lord and Webster, 1979) and *Trifolium pratense* (Heslop-Harrison and Heslop-Harrison, 1983).

The dicotyledonous type of anther development confirmed earlier observations on the Papilionoideae (Davis, 1966). The predominance of tapetal cells, simultaneous cytokinesis and 2-celled pollen grains indicate that *S. formosa* closely resembles other members of the family. The tetrasporangiate type of anther is also common in many other legumes except Carmichaelieae (Goodley, 1980), which has bisporangiate anthers. The uninucleate tapetal cells recorded in many legumes (Davis, 1966; Lim and Prakash, 1997), were also found in *S. formosa*. As with in most members of the family (Prakash, 1987), the anther wall of *S. formosa* shows one endothecial layer and a secretory tapetum. However, in this study only one middle layer was found, whereas in most legumes such as *G. max* and *Psophocarpus tetragonolobus*, two or three middle layers were reported (Prakash and Chan, 1976; Lim and Prakash, 1997).

The morphology and development of the ovule confirms earlier observations on the family (Prakash and Chan, 1976; Prakash and Herr-Jr., 1979; Prakash, 1987; Lim and Prakash, 1997). Ovules in the apical region of the ovary grow faster than ovules in the basal region. A similar pattern of ovule differentiation was also reported in *G. max* (Kato *et al.*, 1954) but Prakash and Chan (1976) found that the basal ovule developed faster than the apical ones. The campylotropous arrangement of ovules, as well as the zigzag pattern of the micropyle formed by both outer and inner integuments are common features throughout the family. This ovule arrangement was also found in the members of

the Malvaceae and Caryophyllaceae (Tootil, 1984). However, as in *G. max* (Prakash and Chan, 1976), the inner integument in *S. formosa* is slow growing and completely covers the nucellus only after fertilisation. Starch grains are abundant in the mature embryo sac of *S. formosa* as in *Arachis hypogaea* (Reed, 1924) and *G. max* (Prakash and Chan, 1976). The presence of starch in the embryo sac seems to be of widespread occurrence in the family (Prakash, 1987).

Although there has been no previous record on the occurrence of multiple embryo sacs in the species, this study revealed that polyembryony could happen in *S. formosa*. Multiple embryo sacs have been reported in a number genera of Mirbelieae, such as *Jacksonia, Dillwynia, Oxylobium, Gastrolobium, Nemcia, Brachysema* and *Jansonia* (Cameron and Prakash, 1994). This finding is the first recorded in the genus so far. A very low incidence of twin seedlings (0.06% throughout the work presented here) suggested that polyembryony is not a common feature of *S. formosa*. This also indicates that although polyembryony could theoretically occur in the ovule, only one mature embryo is usually formed in the seed.

As in most legumes, the endosperm of *S. formosa* is of the nuclear type. However, a considerable variation in the organisation of the endosperm in the family has been recorded in other Australian legumes (Cameron and Prakash, 1994).

The organisation of the seed coat of *S. formosa* is similar to that in most legumes. Corner (1951) described the seed coat of leguminous plants as developing entirely from the outer integument of the ovule through cell division, enlargement and modification of the wall. This study revealed that the testa is typically differentiated into a layer of palisade thick-walled columnar cells on the outside and a mesophyll with a layer of hypodermal sclereids (the so-called hour-glass cells). A similar composition is also noted for the seed coat of *G. max* (Vaughn, 1970; Prakash and Chan, 1976). However, in *P. tetragonolobus* the layer of hour-glass cells is not distinguished in the mesophyll of the seed coat (Lim and Prakash, 1997). The inner integument does not take part in the

formation of the seed coat and is usually crushed in the mature seed (Prakash, 1987).

6.5 Conclusion

In general, floral morphology and floral anatomy in *S. formosa* are typical of leguminous flower. The presence of stigmatic cuticle is another characteristic of papilionoid legumes.

The anatomy and the development of the anther and ovule also reflect the characteristics of leguminous plants. However, unlike most legumes, *S. formosa* is found to have polyembryony though the incidence is very small. The epigeal type of seed germination is a common feature in many legumes.

Further investigation is necessary to complete the picture of detailed embryology and seed development in *S. formosa* as a fundamental aspect of its breeding programme.

CHAPTER 7
POLLEN LONGEVITY AND STIGMA RECEPTIVITY

7.1 Introduction

The cross-pollination technique in hybridisation has been practiced for many years in conventional plant breeding programmes. This method requires either natural pollination by the assistance of birds, insects or wind, or artificial hand-pollination. The purpose of this practice is to transfer pollen from the male reproductive organ of one individual to the female reproductive organ of another individual. Hand-pollination is preferable in most breeding programmes since it offers a controlled method of crossing and obtaining homozygous progeny by preventing crossing between unwanted individuals.

The success of either artificial or natural pollination depends greatly upon the readiness of both male and female reproductive organs to undergo fertilisation and subsequent zygote development. This is indicated by the viability of the pollen grains and the receptivity of the stigma. It is often found that in certain plants hybridisation under natural conditions is not possible because maturation of both male and female reproductive organs does not take place simultaneously due to a wide variation in flowering response, e.g. in *Pistacia vera* (Vithanage, 1984) and *Dendrathema grandiflora* (Ikeda and Numata, 1996). In addition, the presence of the stigmatic cuticle in many papilionoid legumes, including *S. formosa*, has become an obstacle to pollination because pollen will not germinate until the cuticle is ruptured, even though the plant is fully self-compatible (Shivanna and Owens, 1989).

For a successful hybridisation programme, a reliable method of pollen storage is required. Under controlled temperature and storage humidity this method has been successfully applied to a wide range of angiosperms (Hong *et al.*, 1999; Craddock *et al.*, 2000; Martinez-Gomez *et al.*, 2000; Metz *et al.*, 2000; Vaknin and Eisikovitch, 2000; Martinez-Gomez *et al.*, 2002; Parton *et al.*, 2002). The preservation of *S. formosa* pollen was first reported by Hughes *et al.* (1991).

It was found that *Clianthus formosus* (syn. *S. formosa*) pollen grains can be stored for up to 11 days in dry conditions at -180°C without losing their viability. However, pollen storage at such a low temperature requires a specific facility such as a liquid nitrogen refrigerator. The procedure is also more complex as preserved pollen needs to be thawed before being used for pollination. In fact, not many laboratories or small-scale commercial nurseries have such a facility. Thus, this experiment sought a simple and economically sound technique for preserving *S. formosa* pollen grains for breeding purposes.

Stigma receptivity is another aspect that plays an important role in successful hybridisation. For fertilisation to occur, not only should pollen grains be in a viable state, but also the stigma should be in a receptive condition. Arathi *et al.* (2002) suggested that stigma receptivity is an important determinant of reproductive success in plants. Unfortunately, the stigma receptivity of *S. formosa* is not well documented.

This research was aimed at studying pollen longevity and stigma receptivity as an initial step in the conventional breeding of *S. formosa*. Understanding these aspects is important to ensure that pollen and stigma are ready to undergo fertilisation when required.

7.2 Experimental work

Plant materials used in this study were routinely grown in the glasshouse. The description of care of plants is given in Chapter 4: General materials and methods sections 4.2.1 through to 4.2.4. Detailed descriptions relating to specific treatments and procedures are described in the following relevant sections.

7.2.1 Experiment 1: pollen longevity

The objective of this experiment was to study the longevity of *S. formosa* pollen under different storage conditions.

7.2.1.1 Materials and methods

This study consisted of three trials, firstly, pollen grains were left on the plant (within the keel) in the glasshouse under natural conditions, secondly, pollen grains were harvested and kept at a low temperature (4ºC) in total darkness and, thirdly, pollen grains were kept in a dry freezer (-10ºC) and in total darkness. In the first trial, pollen grains were taken from 5 umbels of 10 glasshouse-grown plants (designated as replicates) on the day of anther dehiscence, through to 10 days after anther dehiscence, at daily intervals. Each umbel consisted of 5 – 7 flowers. Pollen grains from the flowers of the same umbel were mixed in a vial before viability assessment. In the second trial, pollen grains were harvested from 5 umbels from 10 different glasshouse-grown plants on the day of anther dehiscence, collected, mixed in a vial, and kept in a refrigerator at 4ºC. The method of the third trial was identical to the second trial, except that the pollen grains were kept in a freezer at -10ºC instead of a refrigerator. Pollen collection in all trials was carried out between 9.00 and 10.00 am.

The viability assessment was carried out by germinating pollen on a modified BK medium (Brewbaker and Kwack, 1963) containing 720 ppm $Ca(NO_3)_2{\cdot}4H_2O$, 200 ppm $MgSO_4{\cdot}7H_2O$, 200 ppm KNO_3, 20 ppm H_3BO_3 and 10% sucrose. Pollen was spread evenly on the surface of one drop of this medium on a clean microscope slide. The slides were placed in closed Petri dishes on a layer of moistened filter paper to allow the pollen to germinate. The dishes were kept at room temperature under a light intensity of 50 µmol m^{-2} s^{-1}. No cover glass was used, as free access to air was necessary for the pollen to germinate. Pollen cultures were left for 120 minutes to obtain the maximum germination before assessment. The slides were set up at 10 minutes intervals and the first slide was examined after 120 minutes. Examination of the slide took approximately 10 minutes by which the second slide was ready for examination. This pattern was continued until all slides were examined and this ensured a uniformity of 120 minutes culture for each slide.

The examination of pollen viability was carried out according to the protocol of Kearns and Inouye (1993). Pollen grains were dusted on a drop of

lactophenol-aniline blue on a microscope slide, covered with a cover slip and examined under a light microscope. Dark blue stained pollen grains were recorded as viable, whereas pale blue stained and colorless grains were classified as unviable.

Except for the pollen left within the keel, the water content of the pollen was determined prior to storage at low temperature. Samples of fresh pollen from different plants were collected in a weighing tray and the fresh weight (A) was measured using an analytical balance. Pollen was then left to dry in a dessicator and the dry weight (B) was determined after 24 hours. Pollen water content, which was known to be 6.6 – 7.3% at the time of harvesting, was calculated using the following formula:

$$\frac{A-B}{A} \text{ x } 100\%$$

where: A = fresh weight

B = dry weight.

Each trial was arranged in a Completely Randomized Design, with 10 replicates (plants). There were 10 fields of view (observations) on each replicate. The percentage of pollen germination was determined by counting the number of pollen grains forming tubes, divided by the total number of pollen grains that were found in one field of view under the light microscope. Only pollen grains having a tube length of at least half of the pollen diameter were counted.

7.2.1.2 Results

The viability of pollen grains was initially high at the time of collection (more than 50% pollen germinated in BK medium). If pollen grains were left within the keel of the flowers on the plants in the glasshouse under natural environmental conditions, the viability was still reasonably high (> 50% pollen germination) for up to 4 days after anther dehiscence. The viability dropped dramatically on the 5th day, indicated by the percentage of pollen germination

(less than 40%). A further decrease in pollen viability (less than 20% pollen germinated) was found on the 10th day after anther dehiscence (Figure 41).

However, if pollen grains were isolated from the plants and kept at a low temperature (4ºC) in total darkness, the viability remained high (> 50% pollen germination) for up to 21 days of storage. Prolonged storage for more than 21 days was found to reduce pollen viability (Figure 42).

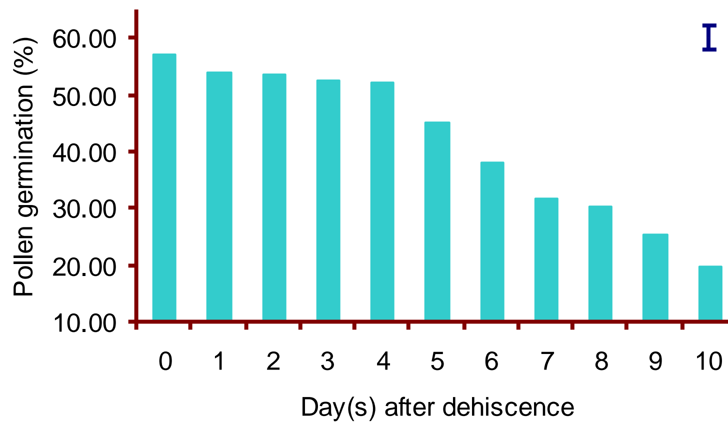

Figure 41.
Germination of *S. formosa* pollen grains from day 0 through to day 10 (after anther dehiscence), when pollen was left within the keel of flowers on plants in the glasshouse (bar represents SE ± 2.17).

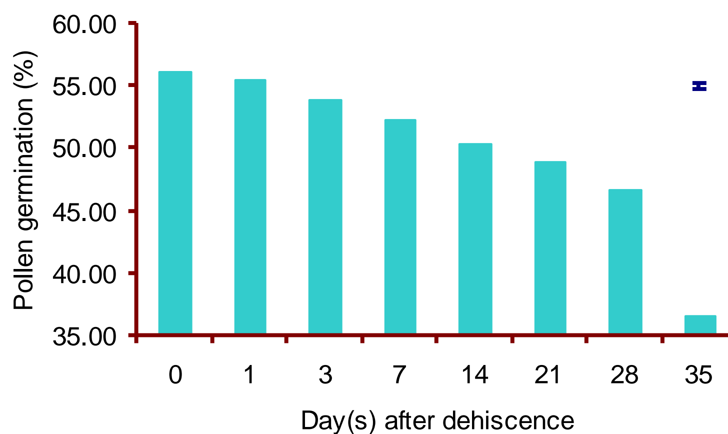

Figure 42.
Germination of *S. formosa* pollen grains from day 0 through to day 35 (after anther dehiscence), when kept at 4ºC in total darkness (bar represents SE ± 0.23).

When pollen grains were isolated from the plants and kept at -10ºC, a different pattern of pollen germination was found. Initially, the rate of pollen germination (58.96%) was similar to that in the trial of low temperature (4ºC) but during the first 3 days of freezing, pollen germination increased up to 73.28%. The rate of pollen germination remained high up to 56 days of storage at -10ºC (Figure 43).

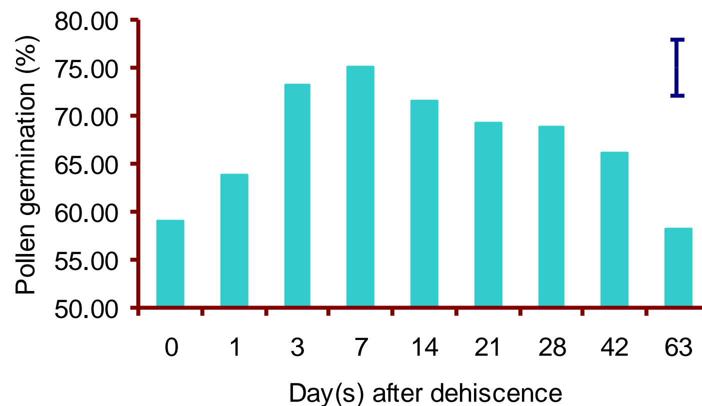

Figure 43.
Germination of *S. formosa* pollen grains from day 0 through to day 56 (after anther dehiscence), when kept at -10ºC in total darkness (bar represents SE ± 2.86).

This study showed that the wall of *S. formosa* pollen consisted of two layers, i.e. the outer and the inner layer (Figure 44A). In certain areas the wall of *S. formosa* pollen was found to be thinner. These regions are known as germinal pores through which the pollen tube may emerge on germination (Figure 44B). Present investigation revealed that *S. formosa* pollen is of tricolporate type, indicated by the presence of 3 pores on each pollen grain. Tricolporate pollen is common in Papilionoideae (Ferguson and Skvarla, 1981).

The examination of pollen viability using lactophenol-aniline blue showed that the viable pollen grains were round in shape and were dark blue stained (Figure 45A-C). Meanwhile, unviable pollen grains had an irregular shape and were pale blue stained (Figure 45D-F).

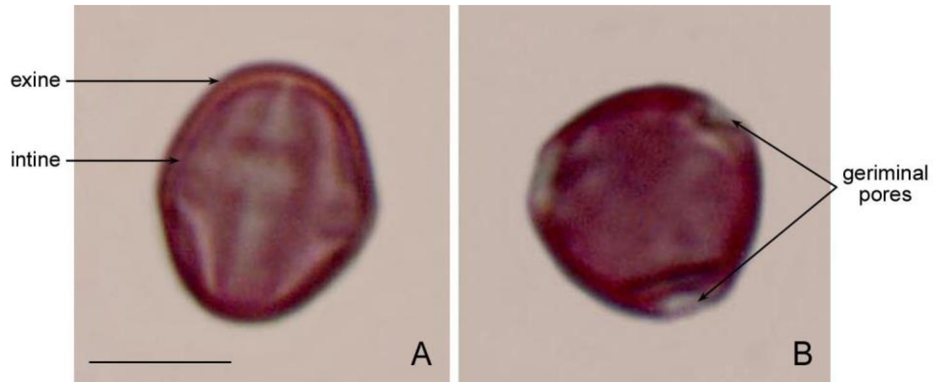

Figure 44.
Pollen grains of *S. formosa*. A, showing exine and intine layers of pollen wall; B, showing 3 germinal pores. Bar = 10 µm.

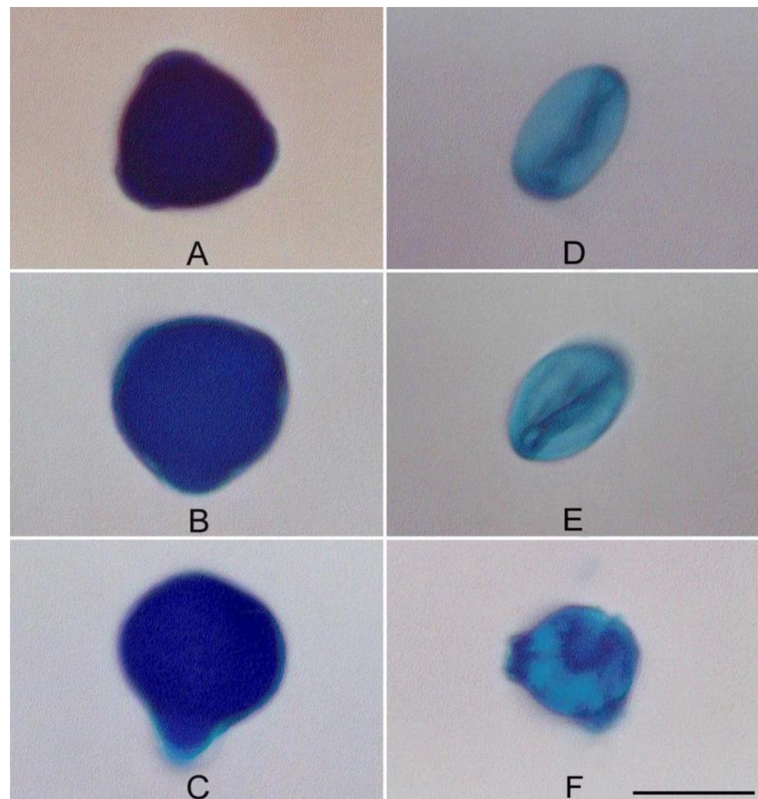

Figure 45.
The difference of viable and unviable pollen grains of *S. formosa* as indicated by lactophenol-aniline blue staining. A-C, viable pollen; D-F, unviable pollen. Bar = 10 µm.

7.2.2 Experiment 2: stigma receptivity

This work sought to ascertain the time at which the stigma of *S. formosa* was most receptive, so that the optimal time for hand-pollination in the glasshouse could be determined.

7.2.2.1 Materials and methods

For practical reasons, flowers were emasculated one day prior to anther dehiscence, or stage 4 according to Jusaitis (1994) (see Chapter 3: The *Swainsona formosa* plant). This was the earliest that the floral buds could be manipulated without causing damage. The emasculated flowers were left unpollinated up to 8 days after anther dehiscence, depending on the treatment. In addition, pollinations were also performed on flowers with just-dehiscing anthers and on flowers with non-dehiscing anthers (on the day of emasculation). The flowers of all treatments were pollinated in one day and this was the same day as the pollination of flowers with just-dehiscing anthers.

The stigma was rubbed several times with a fingertip to remove the stigmatic cuticle and to expose the stigmatic surface. The stigmatic surface was pollinated with fresh pollen grains at the time indicated by the following treatments:

A = flower emasculated 8 days after anther dehiscence
B = flower emasculated 7 days after anther dehiscence
C = flower emasculated 6 days after anther dehiscence
D = flower emasculated 5 days after anther dehiscence
E = flower emasculated 4 days after anther dehiscence
F = flower emasculated 3 days after anther dehiscence
G = flower emasculated 2 days after anther dehiscence
H = flower emasculated 1 days after anther dehiscence
I = flower emasculated on the day of anther dehiscence
J = flower emasculated one day before anther dehiscence.

In order to minimise the experimental error due to environmental variation, all the pollen grains were collected from just-dehiscing anthers of approximately

100 flowers, mixed in a vial and tested for their viability prior to pollination. The pollen viability test indicated that the germination rate on BK medium was between 60 to 65%.

Three umbels (one from each stem) consisting of 5 – 7 flowers were selected as floral samples for emasculation. There were 4 replicates (plants) for each treatment. The number of pods formed was used as the parameter of stigma receptivity in this trial.

<u>7.2.2.2 Results</u>

The results indicated that one day before anther dehiscence the stigma has already become receptive (Figure 46). The stigma receptivity reached its peak on the 2nd and the 3rd day after anther dehiscence, indicated by 100% pod formation, and gradually started to decrease from the 4th day. On the 8th day after anther dehiscence, the petals started to degenerate and the stigma receptivity decreased significantly. On the 9th day after anther dehiscence, flowers were too flaccid to be pollinated. This was the time at which the abscission layer at the pedicel-peduncle junction had developed to the point that abscission was inevitable. At this stage the stigma had completely lost its receptivity.

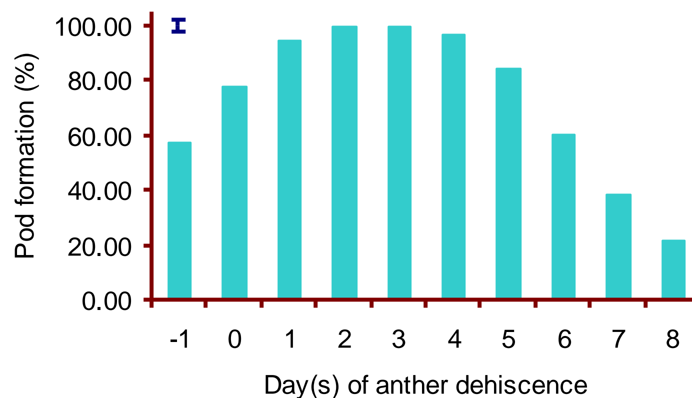

Figure 46.
Stigma receptivity in relation to the timing of anther dehiscence in emasculated glasshouse-grown *S. formosa* flowers (bar represents SE ± 2.35). Day -1 means pollination was carried out one day before anther dehiscence.

7.3 Discussion

7.3.1 Pollen longevity

It has been known that *S. formosa* is able to flower all year round, although for some reason there may be a preferred flowering time in the breeding programme. Because of differences in flowering time, cross-pollination for hybrid production may require the storage of pollen for a certain period of time until receptive stigmas are available. This study showed that in glasshouse conditions *S. formosa* pollen may be left within the keel for a short period of no longer than 4 days before the viability is significantly reduced. However, in the breeding programme, longer pollen longevity is usually needed. This requirement could be met by keeping the pollen grains in dry conditions at 4°C and in total darkness. With this method pollen retained its viability for up to 28 days with a reasonably high germination rate. Pollen longevity was even greater with storage at -10°C in total darkness. It is believed that *S. formosa* pollen might stand freezing conditions for more than 2 months.

The ability to tolerate freezing suggests that storage at low temperature may maintain pollen longevity in *S. formosa,* which is beneficial in breeding programmes. Hughes *et al.* (1991) claimed that pollen of *S. formosa* was classified as desiccation-tolerant. Pollen that is classified as desiccation-tolerant can usually survive low temperatures if it is first dried to a relatively low water content (< 20%). In this study, the water content of *S. formosa* pollen at the time of storage initiation was 6.6 – 7.3%.

Pollen preservation at low temperature has been tested in many plant species. Martinez-Gomez *et al*. (2002) preserved pollen grains of almond at 0 and 4°C. It was found that at these two temperature regimes pollen longevity could reach 2 months with 80% pollen germination on BK medium. Prolonged storage for up to 12 months significantly reduced pollen germination to between 0 and 50%, depending on cultivars. A reasonably high germination rate was also reported in *Brassica napus* pollen that was stored at 3 and 10°C (Lyakh *et al.*, 1998). Pollen of *Potentilla nivea* and *Fragaria ananassa* were reported to retain their viability after three and seven years of storage, respectively, at 4°C (Smith *et*

al., 1989). However, with *Pistacia vera*, Vaknin and Eisikovitch (2000) reported that pollen grains that were kept at 4ºC retained their germinability at least for a week, while at -20ºC pollen lost most of its germinability.

The resistance of *S. formosa* pollen to low storage temperatures could be due to the properties of its wall. Two layers, i.e. the outer and the inner layer, were found in the pollen wall of *S. formosa* (Figure 44A). Moore and Webb (1978) suggested that the outer layer of the pollen wall, which is called the exine, is made up of a protective substance known as sporopollenin, and the inner layer, which is called intine, is made up of cellulose and pectin. Raven *et al.* (1999) added that sporopollenin is present in the pollen wall of all plants and is the most resistant biopolymer known. It is clear that the highly protective sporopollenin present at the outer layer, coupled with cellulose and pectin at the inner layer, contributes to the ability of *S. formosa* pollen grains to be resistant to low temperature, thus retaining their longevity.

Unlike pollen stored at 4ºC, those pollen grains kept at -10ºC were able to maintain their viability above 50% for the duration of the experiment (i.e. 63 days). Formation of intracellular ice crystals is believed to contribute to the extended longevity of *S. formosa* pollen at -10ºC. At the time of preservation, the water content of the *S. formosa* pollen was 6.6 – 7.3%, which was sufficient for intracellular ice crystals to build up; this occurs in *Typha latifolia* pollen with 7% water content, and stored at -20ºC (Buitink *et al.*, 1998). The increased longevity of dry pollen due to the presence of intracellular ice crystals is caused by slow molecular diffusion and decreased chemical reactions (Burke, 1996). Therefore, drying or cooling pollen grains into a glassy state might improve long-term storage of *S. formosa* pollen.

The presence of intracellular ice crystals may also cause abrasion of the exine wall of pollen, resulting in a greater uptake of water and nutrient by the intine during germination. The rate of pollen germination in *S. formosa* increased after several days of preservation at -10ºC, however, as the pollen underwent aging its viability slowly decreased. Further investigations, perhaps using scanning electron microscopy, on the preserved pollen grains would possibly

provide more clues about the effect of low temperature leading to an insight on pollen longevity. This experiment showed that some non-germinating pollen grains were also found to be viable (Figure 45) upon testing with lactophenol-aniline blue. Perhaps these pollen grains would have germinated if the time of incubation of pollen grains on the germination medium increased beyond 120 minutes.

7.3.2 Stigma receptivity

Stigma receptivity is critical in the development of a flower to maturation. It may greatly influence the rate of self-pollination, pollination success at different stages in the flower life cycle, the importance of various pollinators, the interference between male and female functions, the rate of competition via improper pollen transfer, and the chances of gametophytic selection (Galen *et al.*, 1987). Therefore, a test on the timing and duration of stigma receptivity should accompany breeding experiments or artificial pollination procedures (Stone *et al.*, 1995). Receptivity may be influenced by some factors such as the age of the flower and the presence or absence of stigmatic exudate. Generally, the duration of stigma receptivity in most plant species varies from a few hours up to 10 days (Dafni, 1992).

Judging from the fairly high percentage of pod formation (57.72%) in this study it was assumed that the stigma of *S. formosa* had already been receptive for several days before anther dehiscence. However, Jusaitis (1994) claimed that the most convenient time for emasculating and performing hand-pollination was when the petals were still closed but easily opened using the fingers. This stage of floral development was approximately one day before anther dehiscence. Therefore, the earliest time for practically emasculating the flowers and carrying out hand-pollination in the present trial was one day before anther dehiscence. If emasculation is carried out earlier, it will damage the floral parts, particularly petals, because the flowers are still tightly closed and hard to open.

The receptivity of the stigma of *S. formosa* lasted up to the 6th day after anther dehiscence, as indicated by a reasonably high percentage of pod formation

(60.43%). Pod formation significantly reduced on the next day, and no pods were produced through the pollination of stigma on the 9th day after anther dehiscence as the stigma had completely lost its receptivity due to the initiation of floral abscission.

Receptive stigmas are characterised by high enzymatic activity. The presence of several enzymes such as dehydrogenases, esterases and peroxidases is found to coincide with this developmental stage (Shivana and Rangaswamy, 1992). The stigma contains receptive cells that recognise the pollen grain and provides the substrate for pollen germination (Dafni, 1992). It also plays an important role in the selection of pollen, rejecting pollen of alien species as well as fungal spores (Heslop-Harrison, 1999).

The receptive stigmas of *S. formosa* are soft when rubbed with the fingertips because of the presence of liquid exudate. This wet stigma is a characteristic of the Fabaceae (Dafni, 1992). The exudate of the stigmas is important in pollen-pistil interaction, particularly in controlling pollen adhesion, hydration and germination, as well as providing nutrition to the pollen grain during its growth.

One important stigmatic feature in relation to the success of fertilisation is the osmotic pressure (OP) of stigmatic papillae. In self-incompatible plants such as *Linum grandiflorum,* the ratio of pollen OP to stigma OP was different (Murray, 1986). The difference in OP may be the reason for the failure of the pollen to hydrate, or may cause the pollen tube to burst, resulting in the failure of fertilisation even though the stigma is receptive. Since *S. formosa* is self-compatible the OP of stigma should not be a problem in interacting with the OP of viable pollen grains.

Another important element is the morphological structure of the stigma. Heslop-Harrison (1999) suggested that the stigmatic surface of angiosperms shows a wide range of variation. It was reported by Jusaitis (1994) that the stigma of *S. formosa* is covered by a stigmatic cuticle that prevents pollen germination until ruptured (Figure 47A-B). Such rupture can be induced by subjecting the cuticle to mechanical disturbance or increasing fluid pressure in the style (Lord and Heslop-Harrison, 1984) resulting in the rupturing of the cuticle. Although it

may not directly correlate to stigma receptivity, misleading predictions of stigma receptivity in *S. formosa* may occur due to the presence of this cuticle.

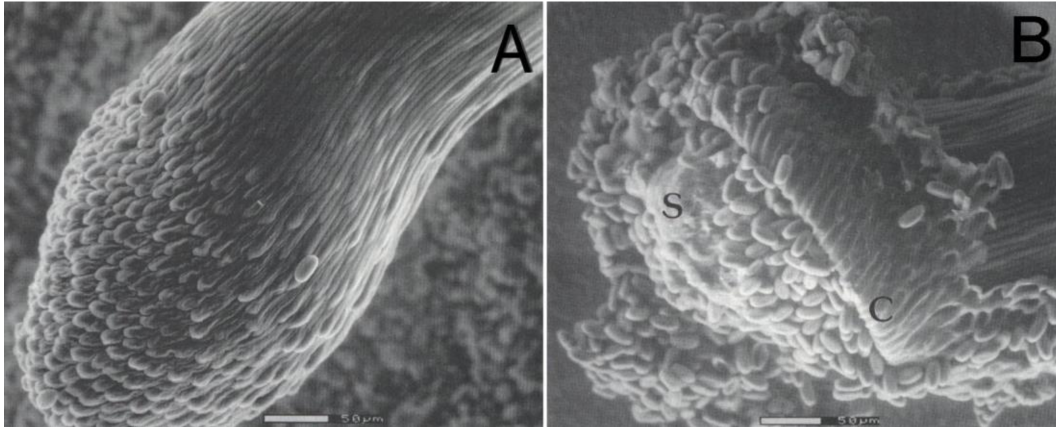

Figure 47.
Scanning electron micrograph of *S. formosa* stigma at floral stage. A, undisturbed stigma showing cuticle surface structure; B, rubbed and pollinated stigma showing the peeled-back cuticle (c) and exposed stigmatic surface (s) (Jusaitis, 1994). (Photos are reproduced with written permission from American Society for Horticultural Science).

7.4 Conclusion

The results indicate that hand-pollination in *S. formosa* could be performed at one day before anther dehiscence, but should be undertaken optimally 1-5 days after anther dehiscence, at which time the stigma is most receptive. When there are no receptive stigmas readily available for pollination, the pollen grains may be left within the keel in the glasshouse for a period of no more than 4 days. Alternatively, the pollen grains could be stored at a low temperature (4ºC) in total darkness, under which conditions pollen viability could remain high for up to 28 days. A longer period of pollen storage could be achieved by the use of freezing methodology (-10ºC), which allows storage of pollen for up to 63 days without any significant reduction in viability. Use of low temperature methodology should allow storage of *S. formosa* pollen for at least a few years, as noted in other species.

CHAPTER 8
ANTHER CULTURE

8.1 Introduction

Anthers contained in young floral buds may be aseptically isolated and used as plant materials in tissue culture. Immature microspores within these anthers may be induced to grow and give rise to complete plants under favourable conditions. Since microspores are haploid, plants regenerated from microspore cells will also be haploid. Haploid plants have no homologous set with which to pair; the normal pairing of chromosomes during meiosis cannot take place. Consequently, sterile plants or plants with non-functional male sexual organs are produced.

Haploid technology is of significant interest for developmental and genetic research as well as for plant breeding and biotechnology. The utilisation of microspore embryogenesis via anther or microspore culture can generate homozygous lines more rapidly than conventional means (Tomasi *et al.*, 1999). In addition, Taji *et al*. (2002) claimed that this would be in only one generation, while conventional methods need at least five generations. Tolerance to unfavourable conditions such as drought, cold, heavy metals or low nutrients are amongst recessive traits that can be detected promptly in haploid plants. The problems associated with outcrossing and self-incompatibility in some species may also be solved by microspore embryogenesis.

Haploid plant production has been successful in various species of monocotyledons such as *Oryza sativa* (Lentini *et al.*, 1995; Aryan, 2002) and *Triticum aestivum* (Touraev *et al.*, 1996). It has been also successful in dicotyledonous species such as *Brassica napus* (Lichter, 1982), *Populus* sp. (Hyun *et al.*, 1986), *Malus domestica* (Höfer *et al.*, 1999), and *Anemone* sp., *Zantedeschia* sp. and *Delphinium* sp. (Custers *et al.*, 2001). There are also reports on haploid plants regenerated from anther or microspore culture with legumes such as *Medicago sativa* (Zagorska *et al.*, 1997), *Cajanus cajun* (Kaur and Bhalla,

1998), *Lupinus* spp. (Bayliss *et al.*, 2002) and some tree legumes such as *Albizzia lebbeck* (Gharyal *et al.*, 1983) and *Peltophorum pterocarpum* (Rao and De, 1987). Tade (1992) attempted to develop a protocol for anther culture of *Swainsona formosa,* but success was limited. The present study further investigated the potential of *S. formosa* anthers to respond to culture under various *in vitro* conditions.

8.2 Experimental work

Plant materials used in this study were routinely grown in the glasshouse. The description on the care of the plants is given in Chapter 4: General materials and methods sections 4.2.1 through to 4.2.4. The preparation of media and explants was carried out as presented in sections 4.3.1 through to 4.3.2. Detailed descriptions relating to specific treatments and procedures are described in the following relevant sections.

Data from all experiments were analysed using ANOVA. The FPLSD test (Fisher, 1966) was employed to separate the means. In addition, where appropriate, the Standard Error (SE) of means were also determined.

8.2.1 Experiment 1: determining microspore developmental stage

The objective of this experiment was to investigate the correlation of floral bud size with the microspore developmental stage as the first step in *S. formosa* anther culture.

8.2.1.1 Materials and methods

A total of 131 floral buds with sizes ranging from 13 to 16 mm were collected from 60 plants. By using graph paper, the distance from the base through to the top of each individual bud was measured (Figure 48). Ten anthers obtained from one bud were bulked-squashed in a few drops of 1% aceto-orcein stain (Prakash, 2000). Observations were made under light microscopy, and approximately 50 microspores from each bud were examined to obtain a consistent result. The stage of the microspore development was determined based on the presence of a nuclear stage. The stages were mother cell, tetrad, early-

uninucleate, mid-uninucleate, and late-uninucleate and pollen grain (see Figure 8 in Chapter 4: General materials and methods).

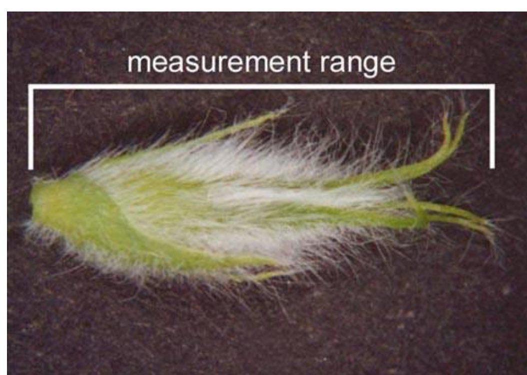

Figure 48.
The measurement range of the floral bud of *S. formosa* to determine the stage of microspore development.

8.2.1.2 Results

The results revealed that there was a strong relationship between the microspore developmental stage and the floral bud length in *S. formosa* ($P < 0.01$). Further analysis using the FPLSD test indicated that the length of the floral buds containing microspores at early-uninucleate, mid-uninucleate and late-uninucleate stages differed significantly from the length of floral buds containing microspores at the tetrad stage. The difference between buds containing microspores at early-uninucleate and mid-uninucleate as well as between mid-uninucleate and late-uninucleate, however, was not significant (Table 6).

Table 6.
The classification of microspore developmental stage in *S. formosa* based on morphological measurements of floral bud length of 131 glasshouse-grown plants.

Stage of microspore development	N	Mean floral bud length (mm)[1)]
Tetrad	50	14.67 ± 0.06 a[2)]
Early-uninucleate	37	15.04 ± 0.07 b
Mid-uninucleate	24	15.21 ± 0.09 bc
Late-uninucleate	20	15.33 ± 0.10 c

N = Number of floral buds examined.
1) = Means ± Standard Error.
2) = Mean separation by FPLSD test at $\alpha = 0.05$.

8.2.2 Experiment 2: the effect of microspore developmental stage

The objective of this trial was to investigate the response of anthers containing microspores at different developmental stages to *in vitro* condition.

8.2.2.1 Materials and methods

This trial used anthers containing microspores of three developmental stages: pre-tetrad, tetrad and post-tetrad. This classification was due to the very short time between the early-, mid- and late-uninucleate stages, while the mother cell and tetrad stages lasted longer and could be easily distinguished. Therefore, pre-tetrad refers to stages starting from the beginning of meiosis until just before tetrad formation, and post-tetrad refers to early-, mid- and late-uninucleate stages plus immature pollen grains. Using floral bud size, microspores of the pre-tetrad stage were predicted to be present in buds 13.5 – 14.5 mm long, tetrads in buds of 14.6 – 14.9 mm long and floral buds 15.0 – 16.0 mm long would contain microspores of the post-tetrad stage.

The composition of the medium used was B5 (Gamborg *et al.*, 1968) basal medium supplemented with vitamins and 2% sucrose at pH 5.8 ± 0.02. Cultures were maintained for eight weeks at a temperature of 25 ± 1ºC and 16-hour photoperiod using cool white fluorescent lamps with light a intensity of 50 µmol $m^{-2} s^{-1}$.

The experiment was a Completely Randomised Design with 10 replicates. Each replicate consisted of 5 Petri dishes containing 10 anthers originating from the same bud. Observations and data collections were made weekly until eight weeks after culture initiation.

8.2.2.2 Results

Anther culture utilising floral buds ranging in size from 13.5 – 15.0 mm long included all the microspore developmental stages (mother cell, tetrad, early-, mid- and late-uninucleate, and young pollen grains). However, unfortunately, no microspore-derived embryos were initiated following the attempted culture. Most anthers were shrivelled with or without callusing after two weeks in culture. The

colour of cultured anthers changed into white, light brown and brown (Figure 49). Browning was also found in callus after two weeks of callus proliferation.

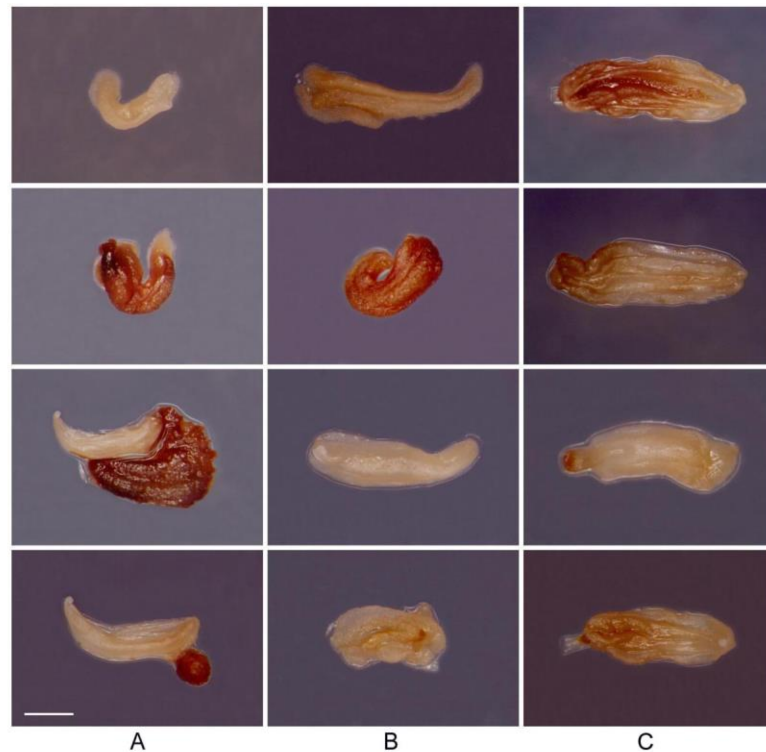

Figure 49.
The appearance of *S. formosa* anthers containing three different microspore developmental stages at eight weeks of culture. A, anthers containing microspores at pre-tetrad stage; B, anthers containing microspores at tetrad stage; C, anthers containing microspores at post-tetrad stage. Bar = 1 mm.

There was no significant difference in the percentage of callus formation on anthers containing microspores at the three different developmental stages. Sixty five percent of callus formation was observed on anthers containing microspores at the post-tetrad stage, followed by 62.5 and 61.43% on anthers containing microspores at the pre-tetrad and tetrad stages, respectively.

Another form of anther response was anther shrivelling. Anthers containing microspores at the post-tetrad stage showed the greatest percentage of anther shrivelling (23.33%) although this was not significant. This was followed by anthers containing microspores at the tetrad (21.43%) and the pre-tetrad (21.25%) stages.

8.2.3 Experiment 3: the effect of media types

This experiment was aimed at studying the response of *S. formosa* anthers cultured on three different media: solid, liquid and paper bridge.

8.2.3.1 Materials and methods

Anthers obtained from young floral buds 15.0 – 15.5 mm long were cultured using the following media: solid, liquid or paper bridge (made from Whatman No. 1 filter paper) on liquid. The median part of the anthers was cut to expose the microspores. Cultures with liquid medium were agitated on an orbital shaker at approximately 80 rpm. All cultures were kept in a growth room at a temperature of 25 ± 1°C with a light intensity of 50 µmol m^{-2} s^{-1} and 16-hour photoperiod using cool white fluorescent lamps.

This trial used a completely randomised design with 10 replicates. Five culture flasks represented each replicate, and 10 anthers were cultured in each flask. Therefore, there were 50 anthers for each replicate making a total of 500 anthers for each type of medium tested. Observations were made weekly and data were recorded for eight weeks after culture initiation.

8.2.3.2 Results

Anthers appeared to be fresh for the first two weeks of culture. Three weeks after initiation, anthers cultured on the solid medium were shrivelled (Figure 50A) and produced white calli, which turned brown one week later (Figure 50B). Anthers cultured in the liquid medium showed limited callus formation but most anthers died after 6 weeks of culture (Figure 50C). Meanwhile, anthers cultured on the paper bridges gradually turned brown at four weeks after initiation with no callus formation (Figure 50D).

The type of culture media significantly affected the response of the cultured anthers ($P < 0.01$). The highest callus formation was obtained for anthers cultured on the solid medium followed by anthers cultured in the liquid medium. Anthers cultured on the paper bridge showed no callus formation (Table 7).

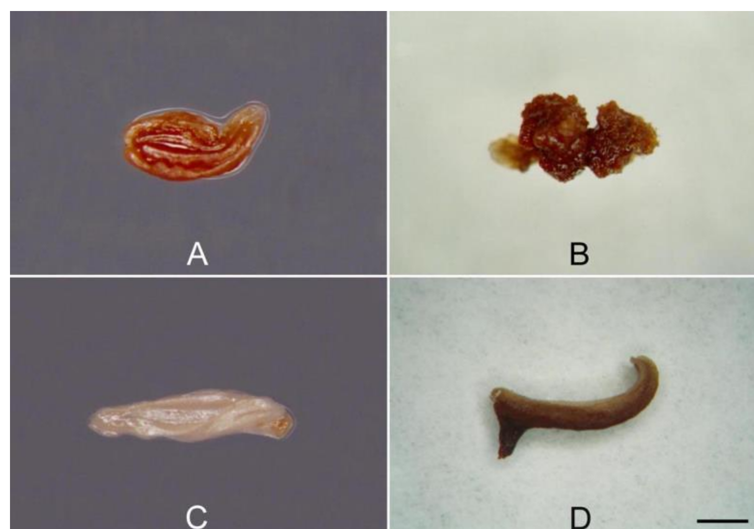

Figure 50.
The response of *S. formosa* anthers on various culture media (8 weeks after initiation). A, brown and shrivelled anther on solid medium; B, brown callus formed on anther on solid medium; C, white anther in liquid medium; D, brown anther on paper bridge. Bar = 1 mm.

Table 7.
The response of *S. formosa* anthers cultured on three different types of media (8 weeks after culture initiation).

Media type	Callus formation (%) [1)]	Anthers' performance
Solid	42.0 ± 3.24 [2)]	Brown and shrivelling
Liquid	16.7 ± 5.12	White
Paper bridge	0.0 ± 0.0	Brown

[1)] Five replicates were used per treatment.
[2)] ± Standard Error of mean.

8.2.4 Experiment 4: the effect of light spectra

The purpose of this trial was to identify the most appropriate light spectra for *S. formosa* anther culture.

8.2.4.1 Materials and methods

Floral buds 15.0 – 15.5 mm long were isolated from glasshouse-grown plants and used as the source of anthers. Anthers were plated horizontally onto the surface of the medium in a plastic disposable Petri dish. The dish was sealed

with a flexible and odourless thermo-plastic parafilm band (Nescofilm®produced by Bando Chemical Ind. Ltd., Japan) to avoid contamination during culture.

Cultures were initiated on the B5 basal medium (Gamborg *et al.*, 1968) as with previous trials and culture flasks were placed on the shelf in the growth room under various light spectra. Six different light spectra were tested: blue (455-500 nm), green (500-560 nm), yellow (565-595 nm), red (640-700 nm) with light intensity ranging from 9 to 14 µmol m^{-2} s^{-1} (depending on the light spectrum) and white (light intensity 50 µmol m^{-2} s^{-1}) at photoperiods of 16-hour, and one continuous total dark condition treatment. The temperature regime under which the cultures were incubated was 25 ± 1°C.

All cultures were arranged in a completely randomised design with 10 replicates. Each replicate consisted of 5 Petri dishes containing 10 anthers each, making a total of 500 anthers for each light spectrum tested. Weekly observations were made and data were recorded until eight weeks after culture initiation.

8.2.4.2 Results

Changes in anther appearance on the anther surface were observed within 10 days after culture initiation. These changes were mainly in the form of callus formation along with shrivelling, particularly on those anthers cultured under continuous dark conditions, as well as anthers cultured under red and yellow light (Figure 51A-C). Anthers cultured under white and green light produced fewer calli but became swollen and green-yellow spots were observed inside the anthers (Figure 51D,E). Meanwhile, anthers cultured under blue light showed no response up to the end of the culture period (Figure 51F).

Light spectra significantly affected callus proliferation for *S. formosa* anther culture (P = 0.01). The highest callus formation (70%) was obtained on anthers cultured under total dark conditions, followed by 58.75, 54.44, 52.22 and 45% callus formation under red, yellow, white and green light spectra, respectively (Figure 52). In contrast, no callus proliferation was found on anthers cultured under the blue light regime.

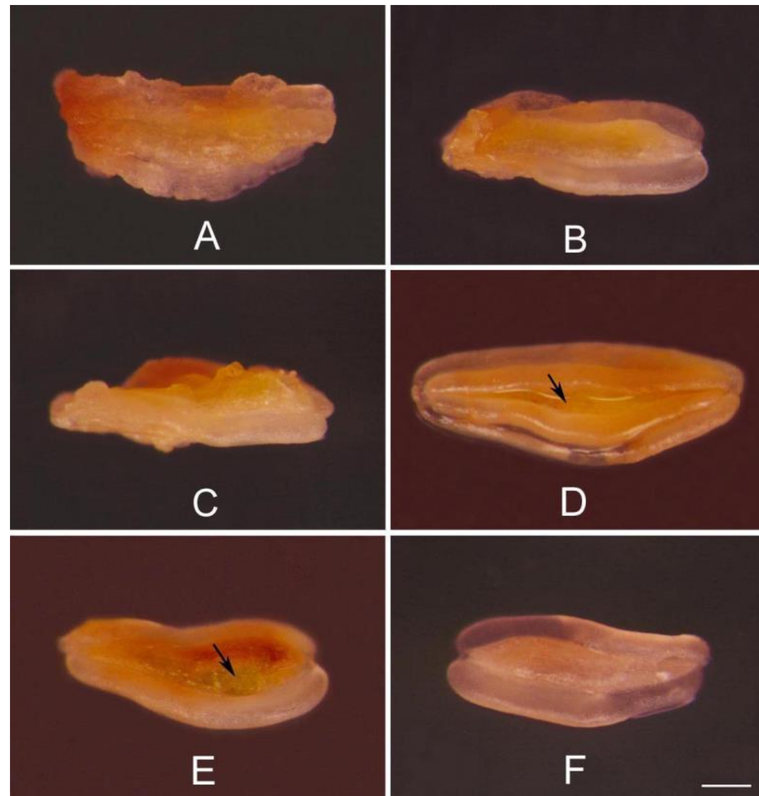

Figure 51.
Response of *S. formosa* anthers cultured under different light spectra. A, callus formed under continuous dark conditions; B, callus formed under red light; C, callus formed under yellow light; D, swollen anther with green-yellow spots (arrow) under white light; E, swollen anther with green-yellow spots (arrow) under green light; F, anther showing no response under blue light. Bar = 1 mm.

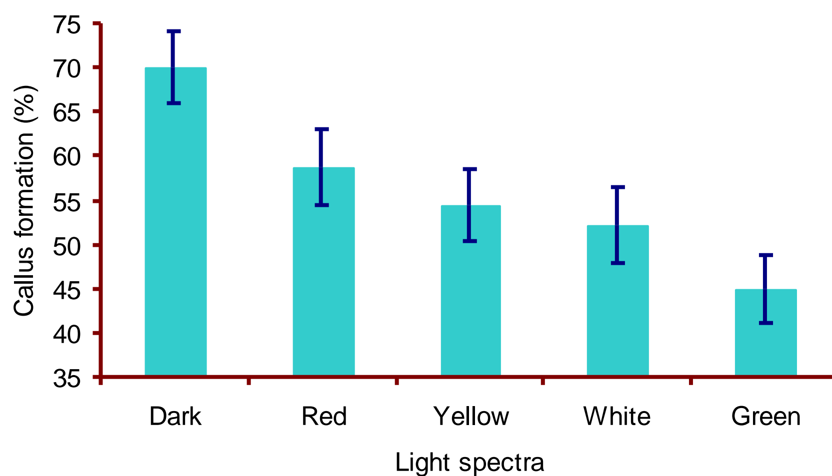

Figure 52.
Callus formation on anthers of *S. formosa* under different light spectra. Bars indicate Standard Errors of means.

8.2.5 Experiment 5: the effect of anther pre-treatment

The aim of this experiment was to test various combinations of stress pre-treatment that were applied to *S. formosa* anthers prior to culture initiation to improve their *in vitro* performance.

8.2.5.1 Materials and methods

Floral umbels with floral buds 15.0 – 15.5 mm long were isolated from glasshouse-grown plants. The peduncles trimmed to about 5.0 cm long, were also included in the isolation. These buds were placed in a vial filled with sterile water or sterile starvation medium. The basal part of the peduncles was in contact with the solution. The vials were kept at a low temperature (4°C) or room temperature (25°C) in total darkness for 2, 4, 6 and 8 days. The starvation medium that was prepared according to Kyo and Harada (1986) contained 1.49 g L^{-1} KCl, 0.12 g L^{-1} $MgSO_4$, 0.11 g L^{-1} $CaCl_2$, 0.14 g L^{-1} KH_2PO_4 and 54.7 g L^{-1} mannitol at pH 7.0.

Following the pre-treatment, anthers were isolated, cultured and maintained in the growth room as in previous experiments. The growth and development of the anthers was recorded for 8 weeks. All treatments consisted of 5 replicates (Petri dishes) containing 10 anthers each.

8.2.5.2 Results

The ANOVA showed that pre-treatment of anthers with starvation at different temperatures for different durations significantly enhanced callus formation (P = 0.04). Pre-treatment with mannitol starvation at 4°C produced more callus than any other treatment at any given duration.

The FPLSD test indicated that at 4°C or 25°C callus formation between water and mannitol starvation was not significantly different at any duration. Significant difference in callus formation was found between mannitol starvation at 4°C and any other three combinations of pre-treatment. The highest percentage of callus formation (98.4 – 98.8%) was obtained with mannitol starvation at 4°C at any given duration. The duration of stress pre-treatment was found to be critical

with mannitol starvation at 25ºC, which showed the percentage of callus formation decreased to 82.6% at 8 days (Table 8).

Table 8.
The effect of temperature and starvation pre-treatments on the percentage of callus formation on anthers of *S. formosa*[1)].

Temperature	Starvation	2 days		4 days		6 days		8 days	
4ºC	Water	93.0bc[2)]	(A)[3)]	91.8bc	(A)	90.4bc	(A)	90.0b	(A)
	Mannitol	98.8a	(A)	98.4a	(A)	98.4a	(A)	98.6a	(A)
25ºC	Water	85.6d	(A)	85.6d	(A)	85.6d	(A)	83.4c	(A)
	Mannitol	92.6c	(A)	89.8cd	(AB)	86.2cd	(BC)	82.6c	(C)

1) Five replicates were maintained for each treatment.
2) Means followed by the same small letter in the same column show no significant difference ($FPLSD_{0.05}$ = 5.90).
3) Means followed by the same capital letter in the same row show no significant difference ($FPLSD_{0.05}$ = 3.73).

Callus was initially formed at the median part of the anthers where the cut was made. This callus proliferated rapidly and within four weeks of culture almost the whole anther was covered with callus. The callus was mostly friable with variation in colour from green on 4ºC pre-treatment to yellow and brown on mannitol at 25ºC pre-treatment (Figure 53).

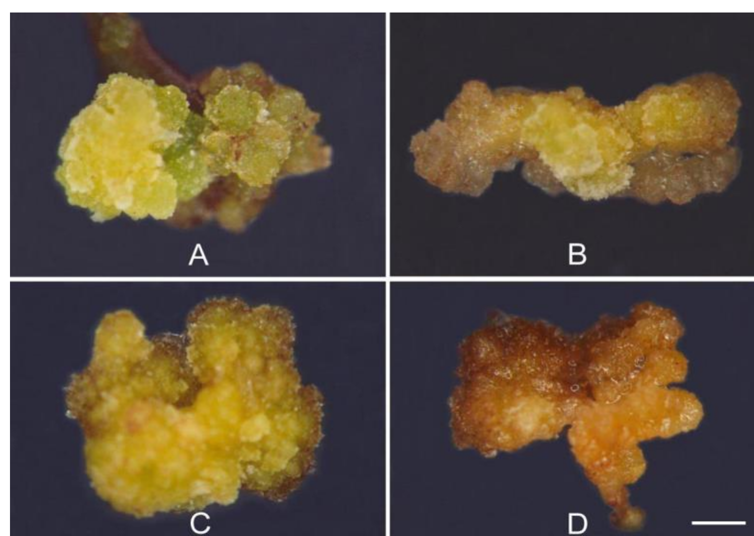

Figure 53.
Callus proliferating on *S. formosa* anthers pre-treated with different temperature and starvation regimes. A, 4ºC without mannitol; B, 4ºC with mannitol; C, 25ºC without mannitol; D, 25ºC with mannitol. Bar = 1 mm.

8.2.6 Experiment 6: the effect of plant growth regulators

The aim of this experiment was to investigate the most appropriate types and concentrations of plant growth regulator (PGR) to use in the medium for anther culture of *S. formosa.*

8.2.6.1 Materials and methods

Total 6 combinations of PGRs were tested for their effect on callus induction. The auxins IAA or IBA were used in combination with either BA, kinetin or zeatin. The concentrations of IAA (0.57, 5.71, 57.1 µM), IBA (0.49, 4.93, 49.3 µM), BA (0.44, 4.44, 44.4 µM), kinetin (0.46, 4.63, 46.3 µM), 2iP (0.49, 4.93, 49.3 µM) and zeatin (0.46, 4.57, 45.7 µM) were tested. One control treatment (growth regulator free) was also included in the experiment. Each of the combinations was tested separately as an independent trial. The experiment was arranged in a Completely Randomised Design with five replicates.

The anthers were cultured on B5 basal medium (Gamborg *et al.*, 1968) supplemented with vitamins, 2% sucrose and PGR according to the combinations described above, for 8 weeks.

The induction of *in vitro* differentiation was attempted from callus that was produced on a medium containing IBA + kinetin and on a medium containing IBA + zeatin. These calli were subcultured onto a fresh solid medium supplemented with similar growth regulators or onto a growth regulator-free medium. The environmental conditions under which the cultures were kept were similar to those described previously (see section 8.2.2).

Three replicates consisting of 3 callus clumps 0.4 – 0.5 cm in diameter were used in this trial. Observations were made for 16 weeks of culture. The means of the number of shoots or roots differentiating from callus on each auxin + cytokinin combination were calculated and the Standard Errors were determined.

8.2.6.2 Results

Callus induction

Callus proliferation was observed 2 weeks after culture initiation in most combinations of auxins and cytokinins. Callus did not proliferate from anthers

cultured on medium supplemented with IAA + kinetin and IAA + 2iP, as well as the control treatment (growth regulator-free medium).

Analysis of variance showed that IAA and BA significantly affected callus formation on *in vitro* culture of *S. formosa* anthers (*P* = 0.01). The maximum frequency of callus formation (26%) was found on culture supplemented with 5.71 µM IAA + 4.4 µM BA, and 57.1 µM IAA + 44.4 µM BA (Table 9). In contrast, IAA + zeatin at different concentrations did not significantly affect callus formation (*P* = 0.80). The data, however, showed that the increasing levels of zeatin tended to produce higher levels of callus formation (Table 10).

Table 9.
The effect of IAA + BA on callus formation in anther culture of *S. formosa*.

Growth regulator concentration (µM)		Average callus formation (%)[1]
IAA	BA	
0.57	0.44	16 a[2]
	4.44	16 a
	44.4	18 a b
5.71	0.44	22 b c
	4.44	24 c
	44.4	26 c
57.1	0.44	18 a b
	4.44	24 c
	44.4	26 c

1) Five replicates were maintained for each combination.
2) Mean values with the same letter are not significantly different ($FPLSD_{0.05}$ = 4.67).

Table 10.
The effect of IAA + zeatin on callus formation in anther culture of *S. formosa*.

Growth regulator concentration (µM)		Average callus formation (%)[1]
IAA	Zeatin	
0.57	0.46	22 a[2]
	4.61	26 a
	46.1	26 a
5.71	0.46	22 a
	4.61	26 a
	46.1	26 a
57.1	0.46	24 a
	4.61	26 a
	46.1	26 a

1) Five replicates were maintained for each combination.
2) Mean values with the same letter are not significantly different ($FPLSD_{0.05}$ = 4.76).

With the use of IBA as an auxin source, the combination of IBA and BA significantly affected callus formation ($P < 0.01$). The highest amount of callus formation (38%) was obtained with the use of 49.3 µM IBA + 0.44 µM BA (Table 11). Similarly, the inclusion of IBA and 2iP in the culture medium significantly affected callus formation in anther culture of *S. formosa* ($P < 0.01$). After 8 weeks in culture, 44.4 µM IBA + 4.93 µM 2iP produced the highest frequency of callus formation (36%) of cultured anthers (Table 12).

Table 11.
The effect of IBA + BA on callus formation in anther culture of *S. formosa*.

Growth regulator concentration (µM)		Average callus formation
IBA	BA	(%)[1)]
0.49	0.44	20 a[2]
	4.44	32 b
4.93	0.44	22 a
	4.44	24 a
49.3	0.44	38 b
	4.44	24 a

1) Five replicates were maintained for each combination. Control, IBA 0.49 µM + BA 44.4 µM, IBA 4.93 µM + BA 44.4 µM and IBA 49.3 µM + BA 44.4 µM were excluded from analysis because the response was nil.
2) Mean values with the same letter are not significantly different ($FPLSD_{0.05} = 4.13$).

Table 12.
The effect of IBA + 2iP on callus formation in anther culture of *S. formosa*.

Growth regulator concentration (µM)		Average callus formation
IBA	2iP	(%)[1)]
0.49	0.44	24 a[2)]
	4.44	24 a
4.93	0.44	24 a
	4.44	32 b
49.3	0.44	24 a
	4.44	36 b

1) Five replicates were maintained for each combination. Control, IBA 0.49 µM + 2iP 49.3 µM, IBA 4.93 µM + BA 49.3 µM and IBA 49.3 µM + BA 49.3 µM were excluded from analysis because the response was nil.
2) Mean values with the same letter are not significantly different ($FPLSD_{0.05} = 4.91$).

A significant effect on callus formation was also found on anthers cultured on the medium supplemented with IBA and kinetin ($P = 0.04$). The maximum

rate of callus formation (36%) was obtained in culture with the use of I 4.93 µM BA + 4.63 µM kinetin (Table 13). Analysis of variance also indicated that callus formation in anther culture of *S. formosa* was significantly affected by IBA and zeatin (P = 0.01). It was found that 0.49 µM IBA + 4.61 µM zeatin produced the maximum frequency (36%) of callus formation (Table 14).

Table 13.
The effect of IBA + kinetin on callus formation in anther culture of *S. formosa*.

Growth regulator concentration (µM)		Average callus formation
IBA	Kinetin	(%)[1]
0.49	0.46	28 a[2]
	4.46	34 a
	44.6	28 b
4.93	0.46	28 a
	4.46	36 b
	44.6	34 b
49.3	0.46	28 a
	4.46	28 a
	44.6	32 ab

1) Five replicates were maintained for each combination. Control was excluded from analysis because the response was nil.
2) Mean values with the same letter are not significantly different ($FPLSD_{0.05}$ = 4.37).

Table 14.
The effect of IBA + zeatin on callus formation in anther culture of *S. formosa*.

Growth regulator concentration (µM)		Average callus formation
IBA	Zeatin	(%)[1]
0.49	4.61	36 b[2]
	46.1	26 a
4.93	4.61	32 b
	46.1	26 a
49.3	4.61	34 b
	46.1	26 a

1) Five replicates were maintained for each combination. Control, IBA 0.49 µM + zeatin 0.46 µM, IBA 4.93 µM + zeatin 0.46 µM and IBA 4.93 µM + zeatin 0.46 µM were excluded from analysis because the response was nil.
2) Mean values with the same letter are not significantly different ($FPLSD_{0.05}$ = 4.91).

Although callus formation appeared to be enhanced by certain combinations of PGR, anthers cultured on media supplemented with IAA + kinetin and IAA + 2iP did not show any response. No response was detected on anthers cultured on

the growth regulator-free medium. Callus was formed on the surface of the anthers and in the region of the cut filament. The properties of the callus varied according to the type of auxins and cytokinins being employed, from non-embryogenic to embryogenic. The non-embryogenic callus was characterised by being spongy and friable in structure, and the colour was white to green (Figure 54A-E), whereas embryogenic callus was nodular and compact and light to dark green in colour (Figure 54F).

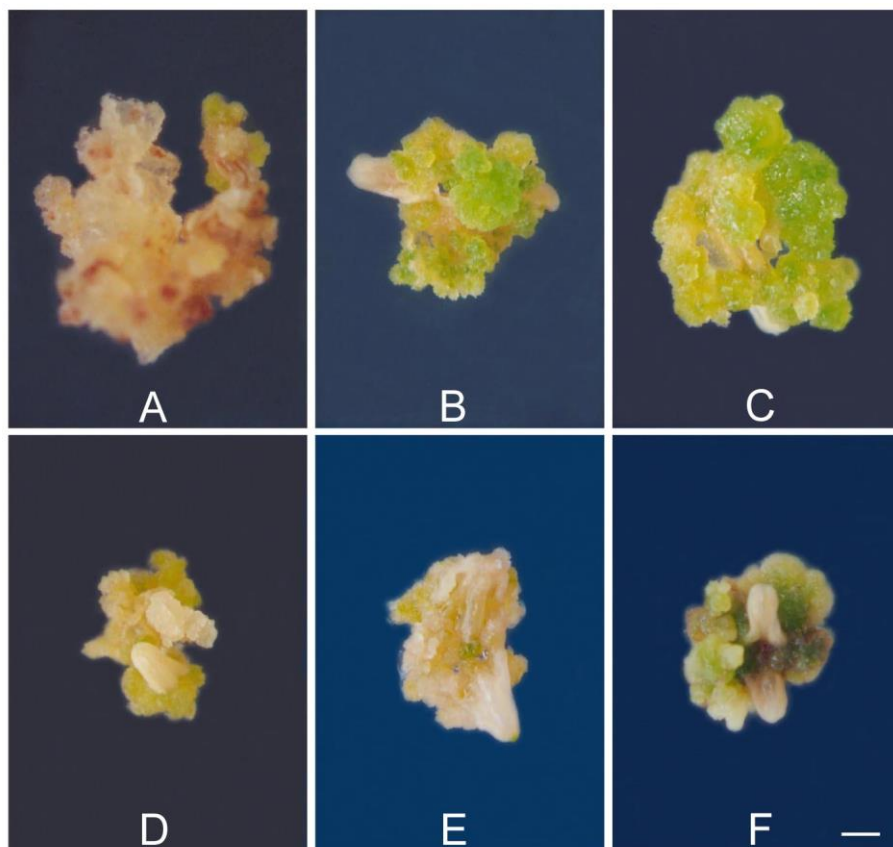

Figure 54.
Some examples of callus formed under different combinations of auxins + cytokinins. A, IAA 57.1 µM + BA 44.4 µM; B, IAA 57.1 µM + zeatin 46.1 µM; C, IBA 49.3 µM + BA 0.44 µM; D, IBA 49.3 µM + 2iP 4.93 µM; E, IBA 49.3 µM + kinetin 4.63 µM; F, IBA 49.3 µM + zeatin 4.61 µM. Bar = 1.0 mm.

In vitro differentiation

Shoot differentiation was found on all callus grown on medium supplemented with IBA + kinetin at all concentrations. Meanwhile, roots were formed on callus cultured on the media containing 4.93 and 49.3 µM IBA in combination with all concentrations of zeatin. However, no treatment concurrently produced both roots and shoots from the same callus. Callus that was transferred onto PGR-free medium showed no further growth, degenerated and eventually died within six weeks of culture (Table 15).

Table 15.
The response of callus following subculture onto fresh medium with or without growth regulators.

Hormone concentrations (µM)			Growth response[1)]	
IBA	Kinetin	Zeatin	No. Shoot[2)]	No. Root[2)]
0.49	0.46	-	7.00 ± 0.57	-
	4.63	-	8.67 ± 0.57	-
	46.3	-	8.50 ± 0.69	-
	-	0.46	-	0.00 ± 0.00
	-	4.61	-	0.00 ± 0.00
	-	46.1	-	0.00 ± 0.00
4.93	0.46	-	3.50 ± 0.69	-
	4.63	-	4.67 ± 0.57	-
	46.3	-	4.33 ± 0.57	-
	-	0.46	-	3.50 ± 0.55
	-	4.61	-	3.33 ± 0.45
	-	46.1	-	3.50 ± 0.55
49.3	0.46	-	3.50 ± 0.69	-
	4.63	-	4.50 ± 0.69	-
	46.3	-	4.50 ± 0.69	-
	-	0.46	-	3.67 ± 0.45
	-	4.61	-	3.50 ± 0.55
	-	46.1	-	3.67 ± 0.45
No PGR (from IBA + kinetin)[3)]			-	-
No PGR (from IBA + zeatin)[3)]			-	-

1) Three replicates were maintained for each treatment.
2) ± Standard Error of mean.
3) No shoot or root formation from callus cultured on PGR-free media.

Meanwhile, shoots that were successfully induced to differentiate from callus subcultured onto the medium supplemented with IBA + kinetin showed

symptoms of hyperhydration. These shoots were characterised by thick leaves and a translucent appearance and showed a very slow growth rate (Figure 55). Following 12 weeks in subculture all shoots showed chlorotic and necrotic symptoms and eventually died 4 weeks later (Figure 56).

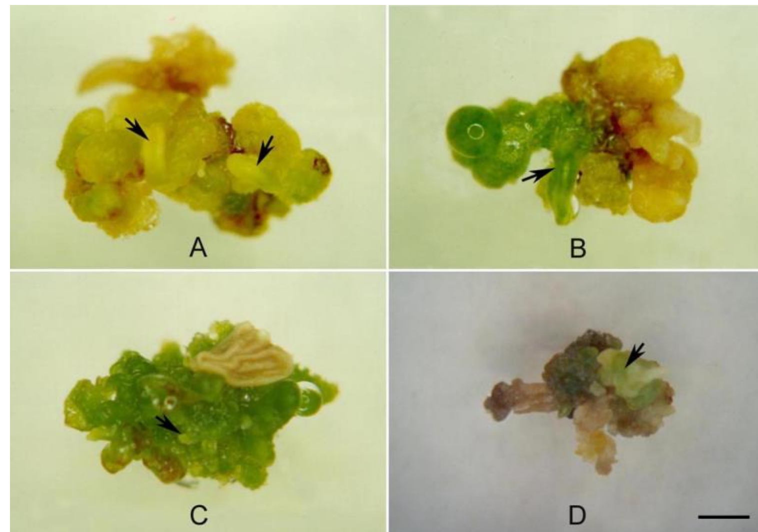

Figure 55.
Representative shoots differentiating from anther-derived callus in *S. formosa*. A,B, shoots (arrows) differentiated on callus cultured on medium containing 0.49 µM IBA + 0.46 µM kinetin; C, green meristematic agglomerates produced on callus cultured on medium containing 0.49 µM IBA + 4.63 µM; D, shoots (arrow) differentiated on callus cultured on medium containing 0.49 µM IBA + 4.63 µM. Bar = 1 mm.

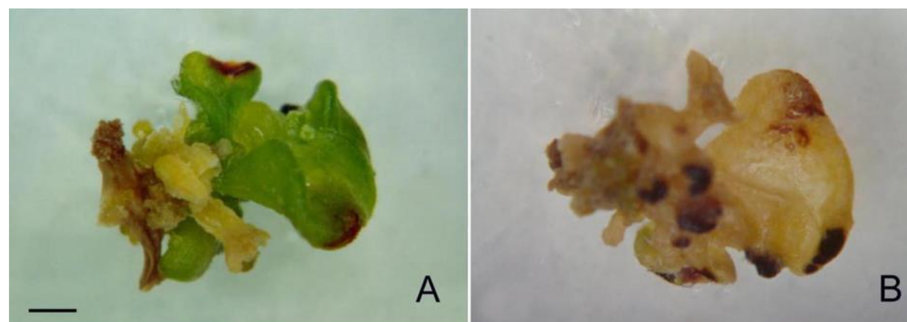

Figure 56.
Necrotic shoots on anther-derived callus of *S. formosa*. A, after 12 weeks of subculture; B, completely degenerated 4 weeks later. Bar = 1 mm.

Instead of forming shoots, callus cultured on fresh medium containing IBA + zeatin showed only root formation. Roots differentiated from callus cultured on medium supplemented with 49.3 µM IBA + 0.46 µM zeatin were thick and short in appearance and without root hairs (Figure 57A). Meanwhile, roots differentiating from callus cultured on the medium containing 4.93 µM IBA + 0.46 zeatin showed a normal appearance with numerous root hairs (Figure 57B).

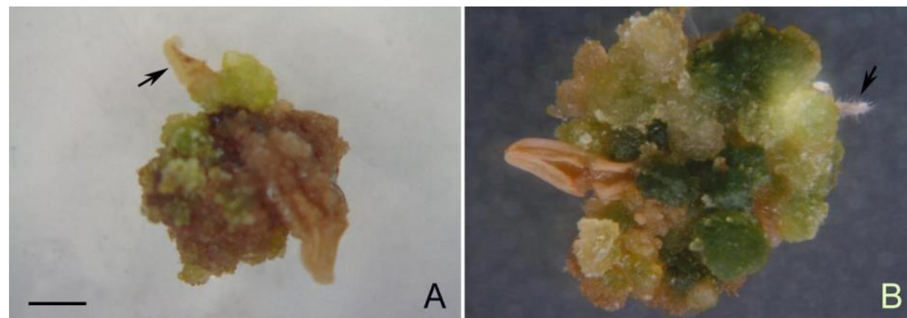

Figure 57.
Roots on anther-derived callus of *S. formosa*. A, thick and short root without root hairs (arrow) differentiated from callus cultured on medium supplemented with 49.3 µM IBA + 0.46 µM zeatin; B, normal root (arrow) differentiated from callus cultured on medium containing 4.93 µM IBA + 0.46 zeatin.
Bar = 1 mm.

8.2.7 Experiment 7: the effect of Ficoll-400™

The objective of this study was to test the effect of various concentrations of Ficoll-400™ (or Ficoll) at different stress pre-treatments on anther culture of *S. formosa.*

8.2.7.1 Materials and methods

For induction, anthers containing microspores at early- to late-uninucleate stages were pre-treated with cold temperature (4ºC) with or without mannitol starvation (factor-1) in similar a way to Experiment 5: the effect of anther pre-treatment. Following isolation from the bud, anthers were plated on solid B5 basal medium (Gamborg *et al.*, 1968) supplemented with vitamins, 2% sucrose and 49.3 µM IBA + 4.61 µM zeatin. Ficoll at concentrations of 0, 5, 10, 15 and 20% (w/v) (factor-2) was applied to the plated anthers to make a double-phase

medium. Cultures were incubated at 25 ± 1ºC and kept in total darkness for 4 weeks but observations were made weekly.

Callused explants were subcultured onto solid B5 medium supplemented with 1% sucrose and 4.63 μM kinetin for embryo induction. Cultures were placed in light with an intensity of 50 μmol m^{-2} s^{-1} and 16-hour photoperiod. Observations for the appearance of somatic embryogenesis were made for another 4 weeks. Somatic embryogenesis was evaluated by counting the presence of embryogenic callus, and embryogenic efficiency (number of embryos per explant) was calculated.

Four weeks later the somatic embryos were transferred to a growth regulator-free medium for maturation and germination. The same light intensity and photoperiod were employed as for embryogenesis.

The experiment was arranged in a Completely Randomised Design with 5 replicates. An ANOVA was employed in data analysis followed by a FPLSD test to separate the means.

8.2.7.2 Results

Callus induction

Analysis of variance showed that Ficoll application significantly affected callus proliferation for anthers of *S. formosa* cultured *in vitro* ($P < 0.01$). A significant effect was also found by subjecting the anthers to stress pre-treatment prior to culture ($P < 0.01$). The interaction of these two factors, however, did not result in significant callus formation ($P = 0.48$). Further analysis using the FPLSD test revealed that significant callus proliferation (67.5 and 45.0%) occurred on anthers pre-treated with either mannitol or water starvation and cultured at 10% (w/v) Ficoll. Ficoll concentrations below or over 10% (w/v) were liable to reduce callus formation either with mannitol or water pre-treatment (Table 16).

As with the other experiments, callus started to proliferate within 2 weeks of culture initiation. Within 4 weeks of culture more calli proliferation was observed on anthers pre-treated with mannitol starvation, particularly those

cultured on 10% (w/v) Ficoll. These calli were white and friable. Following one week subculture on to a fresh medium containing 1% sucrose and 4.63 µM kinetin, and being placed in light (see section 8.2.2), calli from anthers pre-treated with both mannitol and water starvation gradually turned green and grew further resulting in compact to friable structures with or without nodulations and bright to dark green in colour. After 4 weeks of subculture, massive friable callus formations with embryogenic properties were observed, particularly from anthers cultured on 10% (w/v) Ficoll.

Table 16.
The effect of Ficoll concentrations and starvation at 4°C on callus formation on *S. formosa* anthers.

Ficoll concentration (%) w/v	Callus formation (%)1)			
	Mannitol		Water	
0	57.5 abc[2)]	(A)[3)]	32.5 a	(B)
5	62.5 ab	(A)	40.0 a	(B)
10	67.5 a	(A)	45.0 a	(B)
15	47.5 cd	(A)	42.5 a	(A)
20	32.5 d	(A)	30.0 a	(A)

1) Five replicates were maintained for each treatment.
2) Means followed by the same small letter in the same column show no significant difference ($FPLSD_{0.05}$ = 15.32).
3) Means followed by the same capital letter in the same row show no significant difference ($FPLSD_{0.05}$ = 21.66).

Samples of embryogenic and non-embryogenic callus were analysed for the content of amino acid at SARDI Pig and Poultry Production Institute, Nutrition Research Laboratory, Roseworthy Campus, Roseworthy South Australia. The result is presented in Table 17.

In this study, callus formation was associated with localised regions of the anther. Callus was formed mainly on the outer parts of the anther wall that were in contact with the medium. However, it was found that callus also proliferated from within the anthers. Histological examination revealed that callus originated from cells composing the anther wall including endothecium, middle layer and tapetum, but not epidermis (Figure 58A). Callus did not proliferate from microspores (Figure 58B).

Table 17.
Comparison of content of amino acids between embryogenic and non-embryogenic callus derived from anther culture of *S. formosa* (g/kg dry matter).

Amino acids*)	Embryogenic callus	Non-embryogenic callus
Alanine	4.30	4.23
Arginine	4.78	13.09
Aspartic acid	9.00	7.00
Cysteine & Cystine	2.63	2.77
Glutamic acid	9.18	11.06
Glycine	4.91	4.81
Histidine	1.33	2.12
Isoleucine	2.76	4.44
Leucine	5.12	7.11
Lysine	5.02	7.07
Methionine Oxidised	0.98	0.95
Phenylalanine	3.46	5.40
Proline	4.78	not detected
Serine	6.24	7.32
Threonine	3.96	5.12
Tyrosine	3.51	6.37
Valine	5.08	6.93

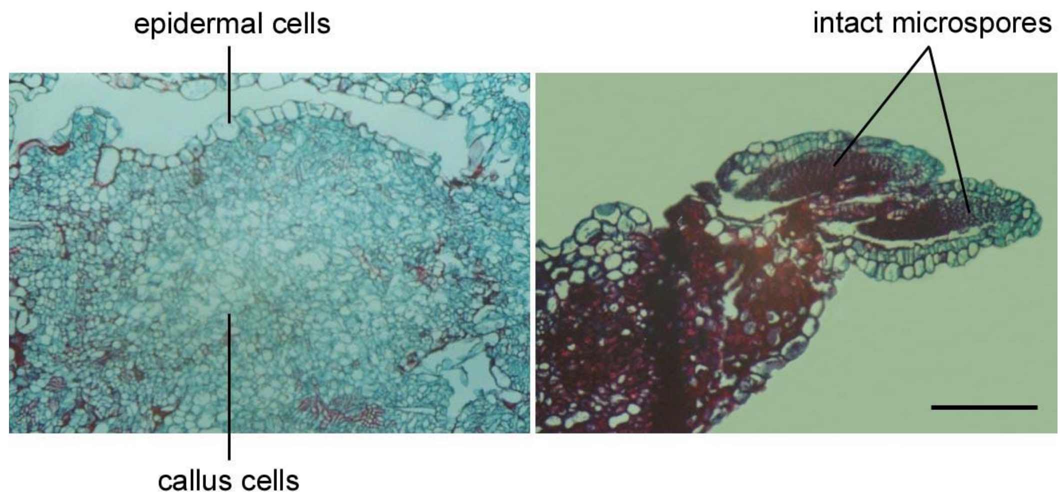

Figure 58.
Histological examination of callus proliferation from anther culture of *S. formosa* four weeks after culture initiation on double-phase medium supplemented with 10% (w/v) Ficoll. A, callus proliferation from anther wall; B, microspores remain intact. Bar = 100 µm.

Somatic embryogenesis

Following subculture on to the medium supplemented with 1% sucrose and 4.63 µM kinetin for embryo induction, somatic embryos were observed within 3 weeks of culture. Embryogenesis was significantly affected by Ficoll concentration (P = 0.02) but not by the starvation treatment (P = 0.15). The interaction between Ficoll concentration and starvation treatment did not show a significant effect (P = 0.94). The FPLSD test showed the highest embryogenic efficiency (1.38 embryos per anther) recorded on callus originated from anthers pre-treated with water starvation and cultured on 10% (w/v) Ficoll was significantly different to embryogenic efficiency from other Ficoll concentrations. Meanwhile, within the mannitol pre-treatment the difference in embryogenic efficiency was not significant at all levels of concentration (Table 18).

Table 18.
The effect of Ficoll concentrations and starvation at 4ºC on embryogenic efficiency from anther-derived callus of *S. formosa*.

Ficoll concentration	Embryogenic efficiency (embryo per explant)[1]	
(%) w/v	Mannitol	Water
0	0.67 a[2] (A)[3]	0.38 a (A)
5	0.58 a (A)	0.44 a (A)
10	1.45 a (A)	1.38 b (A)
15	0.98 a (A)	0.43 a (A)
20	0.61 a (A)	0.23 a (A)

1) Five replicates were maintained for each treatment.
2) Means followed by the same small letter in the same column show no significant different ($FPLSD_{0.05}$ = 0.88).
3) Means followed by the same capital letter in the same row show no significant different ($FPLSD_{0.05}$ = 0.62).

Somatic embryos (Figure 59A-D) were found to germinate and produce normal roots when transferred to the growth regulator-free medium. However, the cotyledons showed symptoms of hyperhydration and no further growth. In addition to hyperhydration, the occurrence of fasciation on somatic embryos was also observed (Figure 59D).

Figure 59.
Somatic embryogenesis on callus derived from anthers pre-treated with mannitol starvation and cultured on double-phase medium with 10% (w/v) Ficoll. A, globular stage; B, torpedo stage; C, early heart stage; D, mature embryo. Bar A-C = 1.5 mm, D = 1 mm.

Shoot and root differentiation

In addition to somatic embryogenesis, shoot and root formation was also found on the callus surface. However, neither starvation pre-treatment nor Ficoll concentrations affected shoot formation (P = 0.53 and 0.26, respectively). No significant effect on shoot formation was shown by the interaction of the two variables (P = 0.98). Within mannitol starvation the highest number of shoot formations was obtained on 10% (w/v) Ficoll, while 5% (w/v) Ficoll produced maximum shoot formation within water starvation (Table 19).

Shoots were found to be hyperhydrated (Figure 60A). Meanwhile, limited root formation (1.5 roots per explant) was only found on callus originated on anthers from water starvation pre-treatment and cultured on double-phase medium with 15% (w/v) Ficoll (Figure 60B).

Different types of callus produced different responses when cultured on medium supplemented with 1% sucrose and 4.63 µM kinetin. For example, green and friable callus produced somatic embryos and bright green callus produced green-coloured nodules (Figure 61) that later developed into adventitious shoots.

Meanwhile, white callus did not turn green but continue to produce a massive callus mass.

Table 19.
The effect of Ficoll concentrations and starvation at 4ºC on shoot and root formation on anther-derived callus of *S. formosa*.

Ficoll concentration	No. shoot formation[1]	
(%) w/v	Mannitol	Water
0	1.00 a[2] (A)[3]	1.25 a (A)
5	1.50 a (A)	2.75 a (A)
10	1.75 a (A)	2.50 a (A)
15	1.00 a (A)	1.75 a (A)
20	0.75 a (A)	1.00 a (A)

1) Five replicates were maintained for each treatment.
2) Means followed by the same small letter in the same column show no significant difference ($FPLSD_{0.05}$ = 2.61).
3) Means followed by the same capital letter in the same row show no significant difference ($FPLSD_{0.05}$ = 1.84).

Figure 60.
Organogenesis from within callus derived from anther pre-treated with water starvation and cultured on double-phase medium. A, 5% (w/v) Ficoll; B, 20% (w/v) Ficoll. Bar = 2 mm.

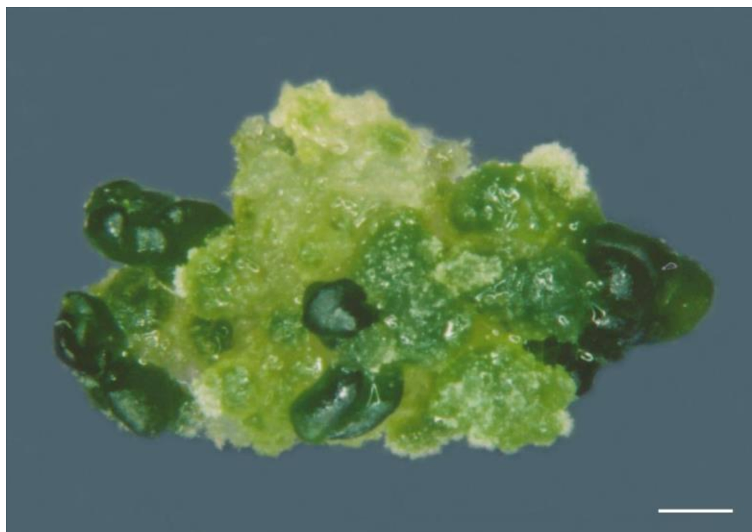

Figure 61.
Bright green callus of *S. formosa* with green-coloured nodules that later developed into adventitious shoots. Bar = 0.5 mm.

8.3 Discussion

Determining the correct stage of microspore development would result in better prediction for the proper timing of culture initiation. Results in this study showed that the stage of microspore development in *S. formosa* was closely related to the size of the floral bud. Therefore, it is not necessary to do a histological assessment (using squash technique staining) on every bud to determine the correct stage of microspore development for culture initiation. A reliable prediction of the developmental stage of microspores can be obtained by measuring the length of floral buds. Tomasi *et al.* (1999) used a similar morphological predictor for determining the microspore developmental stage in *Lasquerella* sp. prior to its anther culture. Floral bud length was then used as a morphological predictor in investigating the effect of the microspore developmental stage; this predictor was also used for subsequent experiments of anther culture of *S. formosa*.

The microspore developmental stage has been known to be a critical factor in anther culture of many plant species but the exact stage for successful plant regeneration is species dependent (Palmer and Keller, 1997). Romeijn and Lammeren (1999) found that first pollen mitosis was a suitable stage for the

induction of androgenesis in *Scabiosa columbaria*. Tetrad to mid-uninucleate stages were found to be useful in androgenesis of *Helianthus annuus* (Coumans and Zhong, 1995; Zhong *et al.*, 1995). Meanwhile, the late-uninucleate to early-binucleate stages were believed to be more responsive in *Brassica napus* (Fan *et al.*, 1988). This study was unsuccessful in inducing plant regeneration in *S. formosa* although all stages of microspore development were tested. This suggests that the success of regeneration in *S. formosa* may also be influenced by other factors, either singularly or in combination.

This study also showed that the response of the anthers were better in solid than liquid or paper bridge media. This could be attributed to the physical properties of the solid medium. In the agar-solidified medium, water and nutrient molecules are incorporated with the agar and this may allow better regulation of nutrient uptake by the cultured anthers, and also better availability of air to the explants. The liquid medium, on the other hand, may provide more contact with water but the lack of density (mass/volume) will cause the anthers to sink. Submerged anthers may suffer from the detrimental effects of anaerobic conditions leading to the death of the tissue (Zhou *et al.*, 1992). Meanwhile, the paper bridge technique is very dependent upon the ability of the paper to absorb water and nutrients (Pierik, 1997). Limited absorption means lack of water and nutrient supply leading to the reduction of growth and development or even to the death of the explants. It could be suggested that the solid medium is necessary for the initiation of anther culture of *S. formosa*.

In addition to the stage of microspore development and media type, anther culture of *S. formosa* showed different responses under various light spectra. For instance, anthers cultured under white and green light produced callus and green tissue, whereas anthers cultured under red and yellow light produced only callus. Meanwhile, blue light showed no effect on cultured anthers. In contrast, conditions of total darkness resulted in more callus proliferation than other light treatments. These responses could be attributed to the presence of phytochrome, a blue-green pigment, in cultured anthers. It is known that phytochrome is responsible for the promotion and inhibition of seed germination in some plants

(Janick, 1986). If microspores are considered similar to seeds, phytochrome could play an important role in signalling an appropriate light spectrum for the induction of microspore embryogenesis.

It is, however, important to note that the response of cultured tissues to light spectra is species-dependent. White or red light enhanced the number of plants regenerating in the anther culture of *Nicotiana sylvestris* (Nitsch, 1981). White and blue spectra were reported to be important in *Melaleuca alternifolia* (Johnson *et al.*, 1996). In addition, Lazzeri *et al.* (1987) reported that both light and dark were crucial in inducing somatic embryogenesis in soybean. In this study the response of anthers cultured under different light spectra was not specific and inconclusive, requiring further investigation.

Stress pre-treatment by mannitol starvation at 4°C was found to be important for callus induction from isolated anthers of *S. formosa*, although this stress might not be sufficient to induce an androgenetic response. Immonen and Antilla (1999) suggested that under stress conditions the normal plant metabolism stopped, and after a certain period of time the resting tissue started out on a new metabolic pathway when introduced to inductive conditions. The response, however, seemed to be species-dependent. In tobacco (Kyo and Harada, 1986) and wheat (Touraev *et al.*, 1996) anther cultures, stress pre-treatment had been found necessary for blocking gametophytic development and triggering pollen embryogenesis, but for recalcitrant species stress pre-treatment alone might be insufficient (Immonen and Robinson, 2000). Van Doorne *et al.* (1995) reported that the recalcitrance of legume tissues caused difficulties in regenerating plants under an *in vitro* system.

The combination of starvation and cold pre-treatment is important to enhance callus induction from anther culture of *S. formosa*. The requirements of starvation and cold pre-treatment has been shown to be important to induce a higher frequency of viable embryogenic microspores in apple (Höfer *et al.*, 1999). It would be necessary to perform further investigations on the effect of anther pre-treatment for plant regeneration from *S. formosa* anther culture.

It was shown in this study that PGR greatly enhanced callus formation on cultured anthers. However, callus that proliferated from the anthers showed different properties depending on the types of auxin and cytokinin. The majority of callus proliferating from anthers cultured on the medium supplemented with IAA + BA were translucent-white and friable. On the other hand, green callus was observed on anther cultured on the medium supplemented with IAA + zeatin. With the use of IBA + BA, a compact yellowish-green callus was produced, while IBA + 2iP produced compact to friable callus coloured whitish-green to dark-green. Meanwhile, friable callus coloured white to green was produced on the medium containing IBA + kinetin, and the medium containing IBA + zeatin produced compact to friable callus coloured light to dark green with some embryonic structures on the surface following subculture onto the new medium.

A comparison of the callusing potential of *S. formosa* anthers exposed to different types and concentrations of auxin and cytokinin indicated that IBA + BA produced the highest callusing capacity (38% on 49.3 µM IBA + 0.44 µM BA). However, embryogenic callus was produced on anthers cultured on the medium supplemented with IBA + zeatin. A similar callus-promoting effect of auxin and cytokinin was reported in previous tissue culture work on *S. formosa* using anthers (Tade, 1992) but no embryogenic callus was produced.

Analysis of the content of amino acids indicated that the embryogenic callus contained 4.78 g per kg dry matter (0.478%) proline. In contrast, no proline was detected in the non-embryogenic callus sample (Table 19). The relationship between the endogenous proline level and callus embryogenetic capacity has also been reported by Murch *et al.* (1999) in *Arachis hypogea*. They found a high level of proline accumulation during the induction phase of somatic embryogenesis from germinated seeds of *A. hypogea* in the presence of thidiazuron (TDZ). The proline content increased significantly throughout the expression phase of embryogenesis. Work by Bella and Shetty (1999) has shown that in *Pimpinella anisum* cultured on medium supplemented with 2,4-D, proline significantly stimulated the embryogenic callus induction and subsequent embryo development on hormone-free medium. The importance of exogenous proline

was demonstrated by Moghaddam *et al.* (2000) on suspension cell culture of sugar beet. It was reported that in the presence of 0.5 mg L^{-1} 2,3,5 triiodobenzoic acid (TIBA), proline treatment at 50 mM increased the frequency of somatic embryogenesis. The results of this study indicate that proline plays a key role in directing the route growth regulator-induced somatic embryogenesis in *S. formosa.* Moreover, the endogenous proline content can be used as an efficient biochemical indicator of callus embryogenetic capacity.

Shoots obtained from callus derived from anthers cultured on medium supplemented with PGR were hyperhydrated and root formation was very poor. Hyperhydration is a common phenomenon in many *in vitro* systems and may be caused by a low medium matrix potential or the accumulation of ethylene in culture vessels. The occurrence of hyperhydration had also been reported on shoots differentiating from callus culture of plants such as *Chrysanthemum morifolium, Rosa hybrida* and *Vitis vinifera* (Smith, 1992) and *Brassica oleracea* (Vandermoortele, 1999). Other phenomena found in this study were the occurrence of chlorosis and necrosis. Taji *et al.* (1995) suggested that chlorosis and necrosis are normally associated with hyperhydrated tissues. Chlorosis and necrosis may occur on regenerated tissues due to a low level of wax in the cuticles (Warren, 1991).

Another way for improving callus proliferation has been culture in a double-phase liquid medium supplemented with a buoyancy increasing component, Ficoll. Experiment 7 showed that the addition of 10% (w/v) Ficoll coupled with mannitol starvation enhanced the number of anthers forming callus, as well as fresh weight of callus per anther. Inclusion of Ficoll improved the density, viscosity and osmolality of the medium (Zhou *et al.*, 1992). In addition, stress pre-treatment of anthers with mannitol starvation stopped normal metabolism and tissues undertook a new metabolic pathway when introduced to *in vitro* conditions (Immonen and Anttila, 1999). Therefore, it can be suggested that the improvement of medium properties coupled with pre-treatment with mannitol starvation resulted in the enhancement of callus proliferation in anther culture of *S. formosa*.

Although Ficoll and mannitol starvation both significantly affected callus formation, somatic embryogenesis from anther-derived callus was only influenced by Ficoll concentrations. A general trend indicated that for both starvation treatments, maximum embryogenesis occurred on 10% (w/v) Ficoll. Ficoll added to the medium coupled with mannitol starvation is an effective way to improve green plant regeneration in triticale (× *Triticosecale*) anther culture (Immonen and Robinson, 2000). Results from the experiments suggest that the combination of mannitol starvation and Ficoll is important for the induction of somatic embryogenesis from anther-derived callus of *S. formosa.* However, as with the findings of Sudhersan and AboEl-Nil (2002), somatic embryos of *S. formosa* showed hyperhydration with limited growth. Further study of the effect of the medium and environmental factors on the growth and development of somatic embryos should be undertaken to obtain complete normal plants.

Callus subcultured onto medium supplemented with 1% sucrose and 4.63 μM kinetin showed different responses. Somatic embryos were produced from green and friable callus and green-coloured nodules, which later developed into adventitious shoots, were produced from bright green callus. Meanwhile, a great amount of callus mass was produced from white callus. Similarly, Tapingkae (1998) reported responses of different *S. formosa* callus types; shoots and roots grew only from green and compact callus, and not from white callus. These findings suggested that chlorophyll plays a key role for initiation of callus differentiation in *S. formosa.*

8.4 Conclusion

S. formosa is normally propagated through seeds and very little is known about micropropagation of this plant. Being a diploid plant, a large number of pollen grains are produced by the flower causing serious problems in the commercialisation of *S. formosa* for cut flowers. The production of haploid plants through anther culture suggests a new approach in breeding strategies of *S. formosa* to produce pollenless flowers. Unfortunately, the present work aimed at producing haploid plants through androgenesis was not successful. However, it is

clear that auxin and cytokinin supplementation, pre-treatment of anthers with mannitol starvation at 4°C and the use of Ficoll in a double-phase medium showed the capacity of promoting embryoid formation from callus. Therefore, future research in anther culture of *S. formosa* should be centred around these variables to induce microspore embryogenesis. Other factors need to be optimised including the spectrum of donor plants used and their growth conditions, as well as the environmental factors within the culture vessels, such as ethylene accumulation and medium pH.

CHAPTER 9
CONVERSION FROM VEGETATIVE TO FLORAL BUDS

9.1 Introduction

One of the key factors influencing success in the induction of polyploidysation using antimitotic chemicals such as colchicine and oryzalin is the age of the plant materials in relation to their life cycle. Most chromosome doubling protocols aimed at producing tetraploid plants were reported to be effective when colchicine or oryzalin was applied during vegetative stage of life cycle of plant (Lucket, 1989; Bruner, 1998; McCuistion and Wehner, 2002). This is because these chemicals will work to inhibit spindle formation and prevent the separation of chromosomes during mitosis only in actively growing tissues.

Colchicine or oryzalin can be applied by soaking the scales, tubers or bulbs of plants prior to culture, or by coating young seedlings in the glasshouse for a certain period of time under conditions of darkness and high humidity. The treatment of scales was found to be effective in doubling the chromosome number in *Cyclamen persicum* (Takamura and Miyajima, 1996). With *Musa* spp., polyploidysation was successfully induced by soaking the sucker in the antimitotic solution prior to *in vitro* culture (Azhar and Rusli, 2000b). However, Bruner (1998), Lucket (1989) and McCuistion and Wehner (2002) claimed that of all the methods of application, shoot apex treatment at the seedling stage was found to be most effective in *Passiflora incarnata, Gossypium hirsutum* and *Citrulus vulgaris*.

It is clear that polyploidy induction should be done during the vegetative stage of plant growth to ensure the production of polyploid cells during subsequent growth. Then, when entering the reproductive stage, the plant will develop male and female gametes that are expected to have 2*x* chromosomes. It is important to note that once conversion to floral apices has occurred, application of colchicine will no longer be effective.

This experiment was aimed at determining the precise time of conversion from vegetative to reproductive growth in *Swainsona formosa* and studying the seasonal influence (photoperiod) that affects this conversion.

9.2 Materials and methods

9.2.1 Experiment 1: glasshouse conditions

This experiment was conducted in the glasshouse with photoperiod ranging from 12 to 16 hours per day and light intensity from 650 to 1200 µmol m^{-2} s^{-1}. The day/night temperatures were set at 30/20ºC. Seed germination and plant care were carried out as described in Chapter 4: General materials and methods. Plant materials were sampled at 14, 28, 35, 42, 49, 56, 60, 64, 65 and 66 days after germination. Four plants were sampled for each treatment.

Shoot tips were collected according to the designated time. Immediately after being isolated from the plants, the shoots were fixed in FPA solution until required. The next step followed the procedure established by Prakash (2000) as described in Chapter 4.

The slides were examined using a light microscope. The microscopic images of shoot apices were captured using a single lens reflex (SLR) camera mounted on the microscope. The pictures were scanned at a resolution of 300 dots per inch (dpi) and the images were computerised for clearer viewing.

At the same time, another experiment was also conducted in the glasshouse. This experiment consisted of 100 plants that were grown and examined for the number of nodes before the first flower. Data were taken from the central stem and two axillary branches growing from the base.

9.2.2 Experiment 2: growth cabinet conditions

In order to obtain a complete picture of seasonal influence (photoperiod) on the conversion of vegetative to reproductive growth, another experiment was set up in the growth cabinets. This experiment was performed in three growth cabinets (Thermoline Australia) each of different photoperiod i.e. 8-, 12- and 16-hour per day, respectively. Light intensity was the same for all cabinets ranging

from 150 to 1000 µmol m^{-2} s^{-1} during the light hours to mimic that of natural glasshouse conditions. The day/night temperatures were set to 30/20ºC for all cabinets. Twenty plants were used as replicates for each photoperiod tested. The care of the plants was similar to that in the glasshouse but the plants were watered manually. The plants were given 250 mL of water at each watering and this was once or twice a day, depending on plant growth. Plant growth and development were observed weekly and data on the number of nodes before the first flower were taken from the central stem and two axillary branches.

9.3 Results

9.3.1 The time of transformation from vegetative to reproductive stage

The histological examination of the shoot apex showed that the plants were still in their vegetative growth stage up to 56 days after germination. Through histological examination it was noted that the conversion from the vegetative into the reproductive stage started on the 60th day after seed germination, when 1 out of 4 sample had the floral apex initiated. On the 65th day after germination all plants had entered their reproductive stage (Table 20).

Table 20.
The status of growth stage of glasshouse-grown *S. formosa* in relation to time after germination.

Plant age (after germination)	Growth stage (%) *)	
	Vegetative	Reproductive
14-days	100	0
28-days	100	0
35-days	100	0
42-days	100	0
49-days	100	0
56-days	100	0
60-days	75	25
64-days	25	75
65-days	0	100
66-days	0	100

*) calculated from 4 plants for each treatment.

In addition to the histological examination, the number of nodes growing before the first flower was initiated was noted for 100 glasshouse-grown plants cultivated at the same time. The results presented in Table 21 show that the central stem grew more nodes before the initiation of the first flower, compared to the axillary branches.

Similarly, experiments conducted in growth chambers with three different photoperiods showed more nodes before the first flower initiation on the central stem than on the axillary branches (Table 22). No significant difference was found in the number of nodes, either on the central stem (P = 0.84) or axillary branches (P = 0.60), among the three photoperiod regimes tested.

Table 21.
Number of nodes before the first flower initiation on the central stem and axillary branches of glasshouse-grown *S. formosa*.

Stems	Number of nodes*)
Central stem	10.55 ± 0.14
Axillary branches	8.11 ± 0.14

*) calculated from 100 plants.
± Standard Error of means.

Table 22.
Number of nodes before the first flower initiation on the central stem and axillary branches of *S. formosa* grown under three different photoperiods.

Stems	Number of nodes according to photoperiod		
	16 hours	12 hours	8 hours
Central stem	12.00 ± 0.22	12.18 ± 0.22	11.85 ± 0.22
Axillary branches	10.50 ± 0.25	10.65 ± 0.25	10.70 ± 0.25

*) calculated from 20 plants.
± Standard Error of means.

9.3.2 Vegetative apex development

The vegetative growth of *S. formosa* was slow at the beginning, particularly during the first 4 weeks after gemination. There was no sign of axillary bud formation during the first 2 weeks (Figure 62) but when the age of the plant

reached 4 weeks after germination the initiation of axillary bud formation was clearly identified (Figure 63).

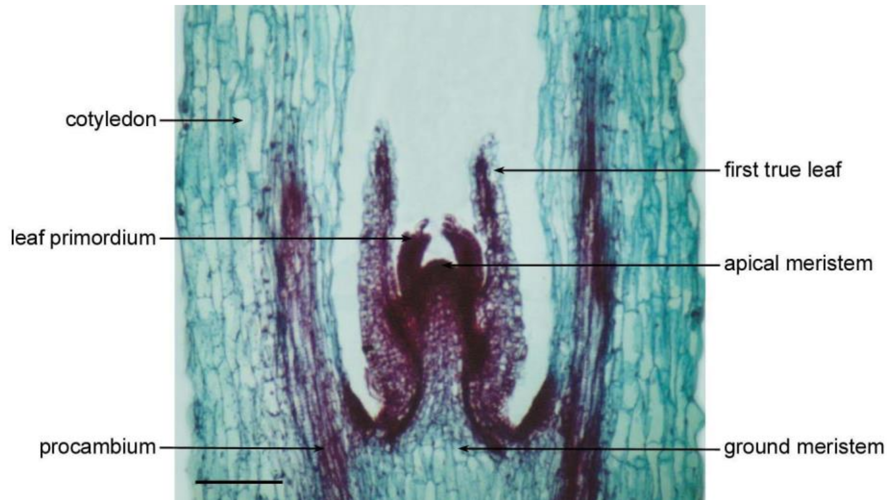

Figure 62.
Longitudinal section of shoot apex of a 2-week old seedling of *S. formosa*. Bar = 100 µm.

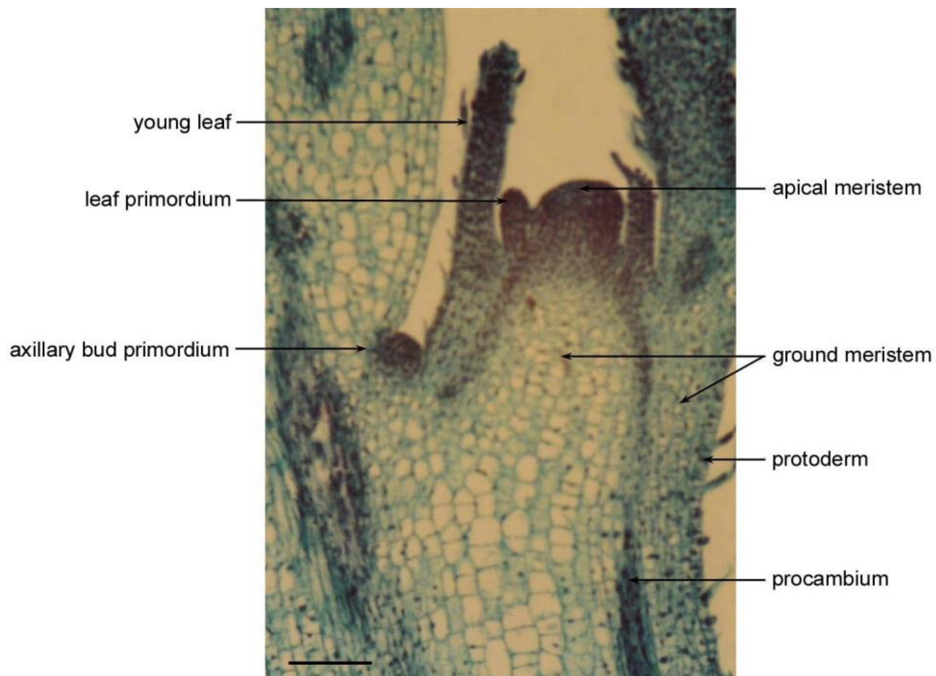

Figure 63.
Longitudinal section of shoot apex of 4-week old *S. formosa* showing the apical meristem and primordia of leaves and axillary bud.
Bar = 100 µm.

9.3.3 Floral apex development

Following the period of vegetative growth, plants entered the reproductive stage during which floral buds were initiated and developed in the leaf axils. This study showed that under the 12- to16-hour photoperiod the transformation from vegetative to floral apex in *S. formosa* started to occur from 60 days after germination (Table 20). Changes were clearly observed when the apical meristem entered the reproductive stage. It was found that the apical meristem grew upward and widened (Figure 64). The tunica and corpus organisaton was still identifiable in the floral meristem (Figure 65) and the layer number in tunica and corpus was the same as in the vegetative stage. Following this, the floral buds developed in the leaf axil where axillary buds normally occurred, and the apical meristem continued to grow upward to form a thick apical dome so that new leaf and new floral primordia were developed (Figure 66).

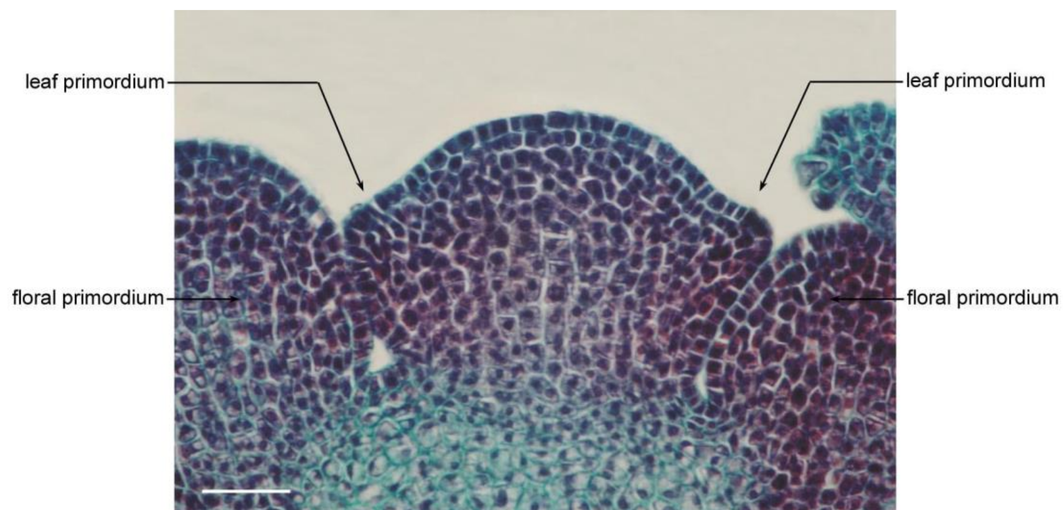

Figure 64.
Floral apex of *S. formosa*. Bar = 25 µm.

Meanwhile, floral buds that arose from the leaf axils continued to grow into a shallow and comparatively broad expanse of meristematic tissue (Figure 67). The broad apex of the floral bud was occupied by a mantle of meristematic cells covering a vacuolated core of ground tissue no longer concerned with upward

growth. The tunica and corpus organisaton was not identifiable in the floral meristem of *S. formosa*.

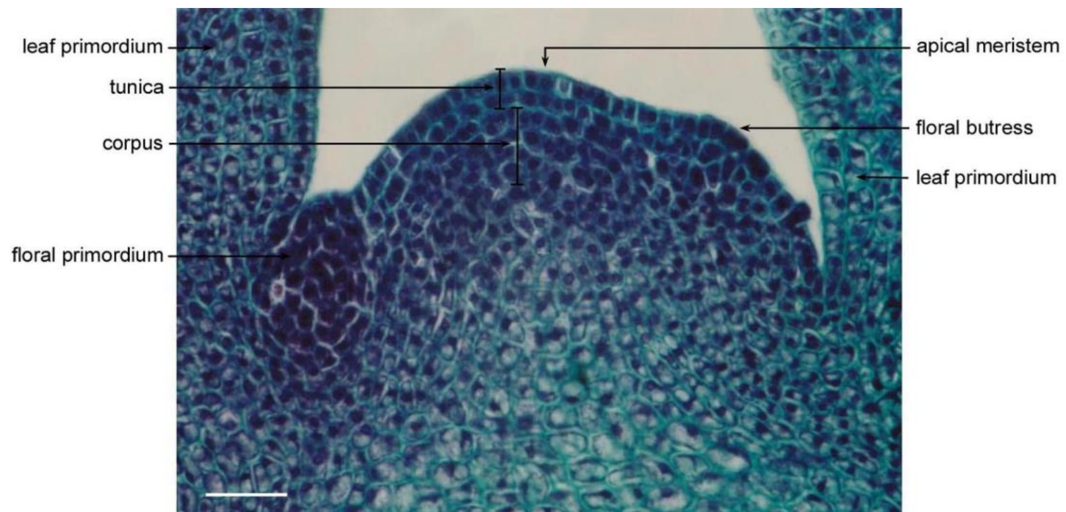

Figure 65.
Longitudinal section of apical meristem of 64-day old *S. formosa* showing tunica-corpus organisaton and the initiation of a floral primordium.
Bar = 25 µm.

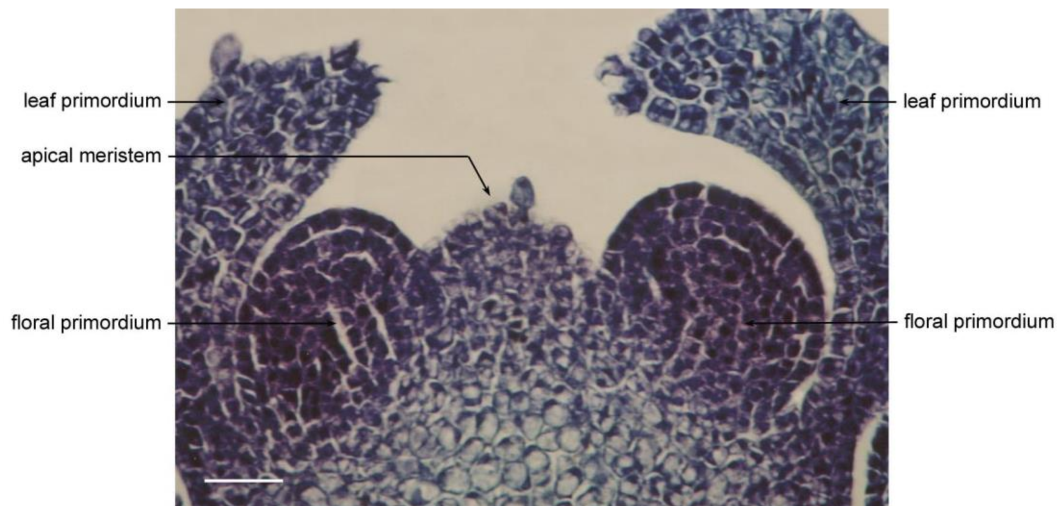

Figure 66.
Longitudinal section of shoot apex of *S. formosa* showing two floral primordia and vegetative apical meristem that continues to grow. Bar = 25 µm.

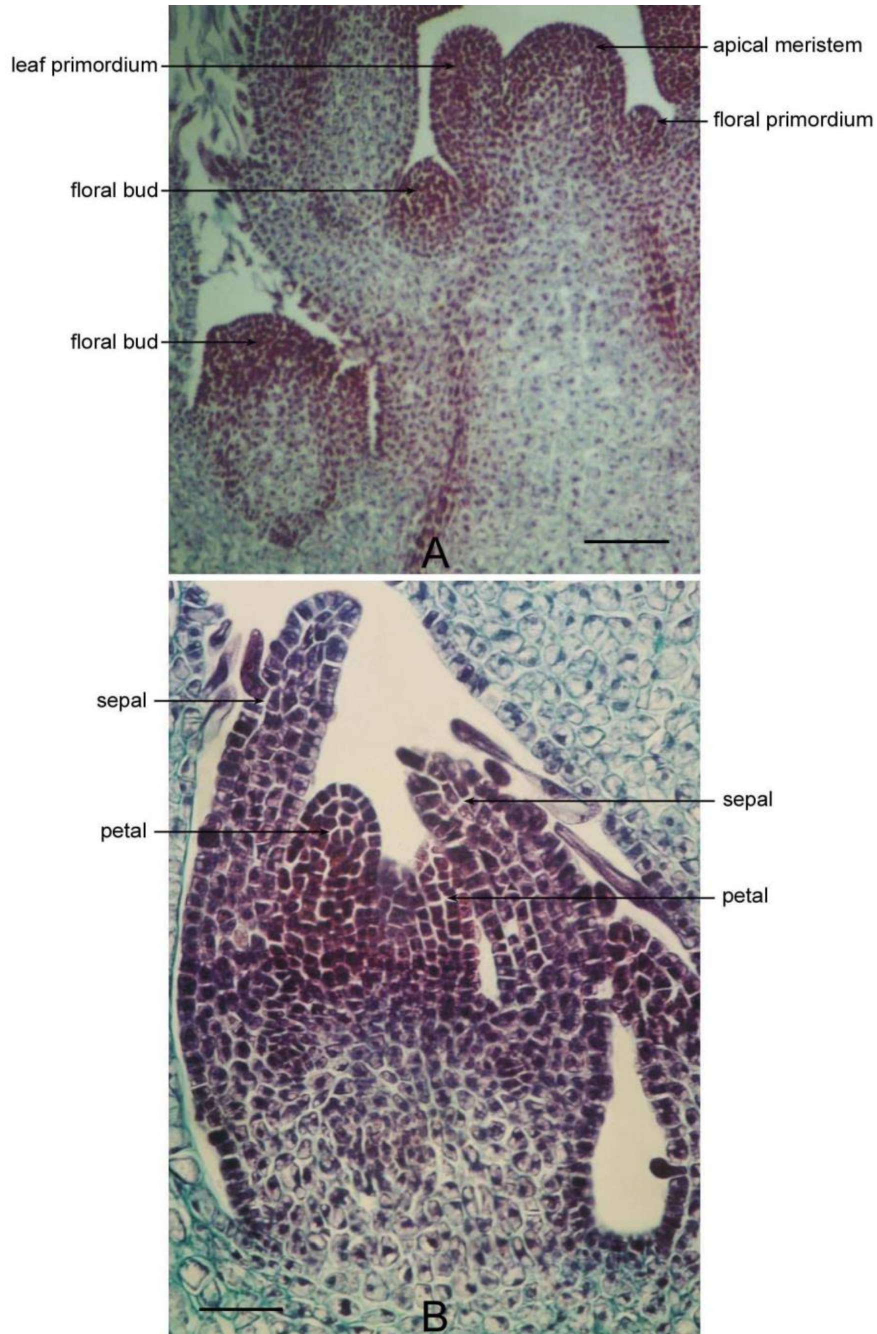

Figure 67.
The development of the floral bud in the leaf axil of *S. formosa*. A, floral buds arising from leaf axil of a 65-day old plant; B, a young flower showing some floral parts. Bar = 25 µm.

9.4 Discussion

Based on its seasonal growth cycle, *S. formosa* is classified as an annual plant. This means that the entire cycle from seed to vegetative stage, from

vegetative stage to flowering and from flowering to seed again occurs within a single growing season. A similar growth cycle is also found in other legumes such as *Glycine max, Cicer arietinum* and *Phaseolus vulgaris*, as well as in other plants such as *Oryza sativa, Hordeum vulgare, Zea mays, Musa* spp. and *Helianthus annuus.*

As with most plants, during its vegetative growth the shoot apex of *S. formosa* produced stems and leaves. A signal such as photoperiod triggered changes in the shoot apex metabolism resulting in the transformation from the vegetative apex into the floral apex and the plant entered the reproductive stage. During this transformation, the floral buttress was initiated, this grew further into a floral bud, and finally into a flower cluster replacing the axillary bud at the leaf axil. Each flower cluster consisted of 5 to 7 flowers (Figure 68) that were borne on a peduncle up to 15 cm long (see Chapter 10: Polyploidy induction).

Figure 68.
Flowers of *S. formosa* at full bloom showing clusters consisting of 5 flowers.
Each flower cluster is supported by a peduncle arising from the leaf axil.
Bar = 3 cm.

Williams (1996) reported that *S. formosa* may start to flower within 70 to 84 days after germination and plants would flower all year round. Yusuf *et al.* (2002) gave a more precise figure on the relationship between photoperiod and first flower appearance on this plant. It was reported that plants grown under a longer photoperiod produced flowers faster than plants grown under a shorter photoperiod. Plants receiving a 16-hour photoperiod flowered within 61 days after germination. On the other hand, plants grown under 12- and 8-hour photoperiods, started to flower on the 66th and 71st day after germination, respectively. The observation of Yusuf *et al.* (2002), however, was based on visual examination of the floral buds. Histological investigations in this study supported their findings and suggested that the period between the 56th and 60th day after germination is critical for the conversion of vegetative growth to reproductive growth in *S. formosa* grown under a 12- to 16-hour photoperiod.

This study showed that although the time required for first flower initiation was influenced by photoperiod, the number of nodes among plants grown under the three different photoperiod regimes was not significantly different. Plants grown under the photoperiods of 16-, 12- and 8-hour per day using artificial light with an intensity of 150 to 1000 µmol m^{-1} s^{-1} produced more nodes, for both central stem and axillary branches, before the first flower initiation than plants grown in the glasshouse. The reason for this difference is due to the delay in the conversion from vegetative to reproductive growth occurring on plants grown in the growth chambers. The delay in growth could be attributed to the constant subtle vibration of the benches due to the air conditioning system in the growth chambers. This subtle vibration may induce stress on the plants (Andersen and Andersen, 2000) resulting in the increase in nodes before flowering. In addition, Taji *et al.* (1995) suggested that plants grown under stress conditions produced large amount of ethylene gas causing inhibition of plant growth and development. As a result, flowering was delayed and the plants developed more nodes before the first flower was initiated.

Although the difference in the number of nodes before the first flower, either on the central stem or axillary branches, on plants grown under the three

photoperiod regimes was not significant, it was clear that more nodes were produced by the central stem. A similar pattern was also recorded on plants grown in the glasshouse. The reason for fewer nodes on the axillary branches than on the central stem is due to the sturdier growth of the central stem. The axillary branches of *S. formosa* grow as plagiotropic branches that have a poorer growth than the central stem (Sudhersan and AboEl-Nil, 2002). This could be due to the difference in hormonal balance, especially ethylene, in plagiotropic shoots. Blake *et al*. (1980) suggested that ethylene was involved in the plagiotropic growth of branches in *Cupressus arizonica* and could indicate changes in auxin or other compounds.

In addition to the critical period (between the 56th and the 60th day after germination) mentioned earlier, this study suggested that node 10 on the central stem and node 8 on the axillary branches are the critical nodes for the conversion from vegetative to reproductive growth in glasshouse-grown *S. formosa.* In addition, node 11 on the main stem and node 10 on the axillary branches are the critical nodes for the conversion from vegetative to reproductive growth on plants cultivated under growth chamber conditions.

The histology of the apical meristem of *S. formosa* followed the common pattern. Beneath the apical meristem, the tissue progressively differentiated through changes in cell size, degree of vacuolation, and rate and orientation of mitoses (Figure 63). Below the apex, was the region of tissue differentiation (histogenesis) and further below this the region of organogenesis such as differentiation of leaf primordium. The peripheral region from which the foliar primordia, epidermis, cortex and vascular tissues originated was distinguishable from the pith region. In the peripheral region the cells remained meristematic, characterised by a relatively small size and less vacuolation. These cells were also longer than those in the pith region.

The apical meristem of *S. formosa* reflected the characteristics of most angiosperms. The cells in the distal part of the apex were arranged in layers known as a stratified meristem (Esau, 1977). Three or four layers growing

independently from one another and two superimposed tiers of initials were present.

9.5 Conclusion

In general, the first flower of *S. formosa* can be observed visually from the 61st to the 84th day after germination (Williams, 1996; Yusuf *et al.*, 2002), depending on photoperiod. Results from the histological study on the shoot apices of plants grown in the glasshouse under a 12- to16-hour photoperiod showed that the time between the 56th and 60th day after germination was the critical period for the conversion from vegetative to reproductive growth in this species. Therefore, it is suggested that any treatment to modify ploidy level in *S. formosa* should be given no later than 60 days after germination.

Another reliable indicator for the conversion from vegetative to reproductive growth in *S. formosa* has been the number of nodes produced before first flower initiation. The number of nodes growing before first flower initiation was not photoperiod-dependent. However, differences in the growth habit between the central stem and axillary branches resulted in more nodes being produced before first flower initiation on the central stem, compared to the axillary branches. The difference was also noted between plants grown under normal conditions (in the glasshouse) and under artificial conditions (in the growth chambers). Artificial conditions were liable to inhibit the growth and development of the plant resulting in delayed flowering. As a consequence, stressed plants produced more nodes before first flower initiation than normal plants. Under normal conditions, 10 and 8 nodes were critical for the central stem and axillary branches, respectively for the initiation of the first flower. Meanwhile, under artificial conditions, the critical nodes were 11 and 10 for central stem and axillary branches, respectively. These critical nodes should be taken into account in the manipulation of ploidy levels in *S. formosa.* Antimitotic agents should be applied to the plants before they reach nodes 8 – 10 depending on the conditions under which the plants are grown.

CHAPTER 10
POLYPLOIDY INDUCTION

10.1 Introduction

The use of triploid hybrids has provided a method to produce sterile plants such as seedless watermelon. In this case crossing a tetraploid ($2n = 4x$) male parent with a diploid ($2n = 2x$) female parent resulted in female-sterile hybrids that are seedless. The reciprocal cross (diploid male parent) will result in male-sterile offspring that produce no pollen grains. Triploid plants have three sets of chromosomes, and three sets cannot be divided evenly when they go into two daughter cells during meiosis. As a consequence, an abnormality in gametogenesis takes place resulting in sterility.

The occurrence of triploid hybrids in watermelon has encouraged the idea of producing male-sterile plants in *Swainsona formosa* using similar techniques. The importance of male-sterile *S. formosa* is that the plants do not produce pollen grains, and therefore the problems of petal staining due to released pollen, as well as quick flower degeneration due to self-pollination can be avoided.

The development of triploid cultivars needs extra time for the development of tetraploids because polyploidy has not always been shown to occur naturally. An antimitotic chemical may be used to induce artificial polyploidysation. The most popular chemical used for chromosome doubling in many crops has been colchicine (Griesbach, 1990; Hamill *et al.*, 1992; Smith and Hamill, 1996; Takamura and Miyajima, 1996). Colchicine acts by disrupting spindle formation and prevents chromosome separation at anaphase during mitotic cell divisions, hence resulting in doubling the number of the chromosomes in daughter cells. Oryzalin also inhibits mitotic activity and is one of the chemicals used for chromosome doubling in *Nicotiana plumbaginifolia* (Varhoeven *et al.*, 1990), *Zea mays* (Wan *et al.*, 1991) and *Lilium longiflorum* (Takamura *et al.*, 2002).

This study attempted to induce chromosome doubling in *S. formosa* using both colchicine and oryzalin. The flowers of treated plants, upon pollination and fertilisation, produce seeds that are expected to grow into tetraploid plants. Crossing diploid and tetraploid individuals will result in the formation of triploid plants with non-functional gametophytes due to abnormalities in gametophytic development.

10.2 Seed treatment with colchicine

10.2.1 Materials and methods

This preliminary study was carried out by treating *S. formosa* seeds with various concentrations of colchicine. Two hundred seeds were soaked in just-boiled water for about 10 seconds, then left in cool water at room temperature for 24 hours. Four concentrations of colchicine solution, 0, 1, 5 and 10%, were used. Fifty swollen seeds were soaked in each concentration for 24 hours in total darkness. Following the colchicine treatment, the seeds were rinsed three times with distilled water to remove the excess colchicine. The seeds were allowed to germinate on double-layered moistened filter paper in closed Petri dishes for another 72 hours before being transferred to Jiffy pots. The Jiffy pots were placed in a shaded glasshouse and watered with intermittent mist spray. The seedlings were allowed to grow to the flowering stage in a temperate glasshouse with similar conditions as outlined in Chapter 4: General materials and methods.

The stock solution of 20% colchicine was prepared by dissolving 2 g colchicine into 10 mL distilled water. The solution was diluted using distilled water to concentrations of 1, 5 and 10%. For the control treatment (0% colchicine) distilled water was used instead of the colchicine solution.

Data were taken for the percentage of seed germination, seedling survival in Jiffy pots and the percentage of plants grown to maturity. Ploidy assessment was made by counting the chromosome numbers in microspore mother cells of treated and non-treated plants. The aceto-orcein method (Prakash, 2000) was used to prepare the material for the chromosome count.

10.2.2 Results

Colchicine treatment of *S. formosa* seeds showed a detrimental effect on germination (Figure 69). The effect was worse at higher concentrations. In addition, the survival rate also decreased with an increase in concentration. Further reduction in survival rate was shown in the number of plants reaching the flowering stage in the glasshouse. Among plants reaching maturity, none was found to be tetraploid (Table 23).

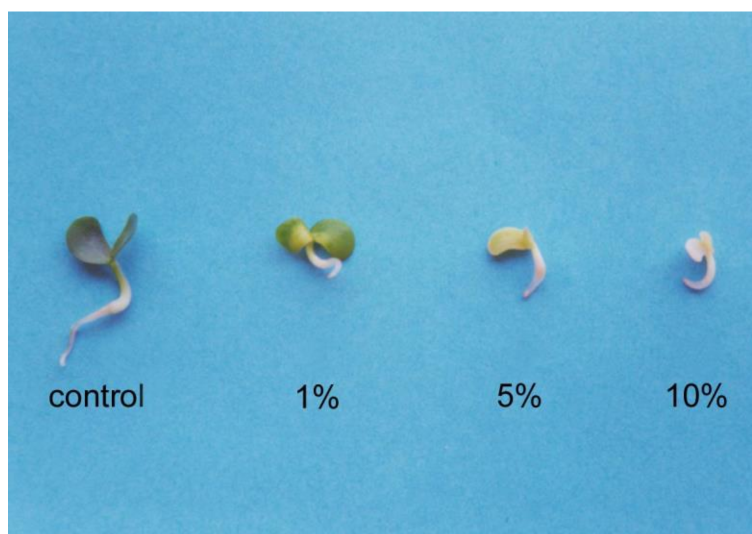

Figure 69.
The effect of different concentrations of colchicine on germination of *S. formosa* seeds.

Table 23.
Effects of colchicine at various concentrations on seed germination, seedling and mature plant survival rate and poplyploidysation in *S. formosa*.

Colchicine concentrations	Germinated*) (%)	Survived in Jiffy*) (%)	Maturity*) (%)	Tetraploid*) (%)
0%	100	100	100	0
1%	40	24	8	0
5%	26	10	0	0
10%	16	0	0	0

*) calculation based on 50 seeds for each concentration tested .

10.3 Seedling treatment with colchicine and oryzalin

10.3.1 Materials and methods

10.3.1.1 Plant materials

Seed germination was carried out according to protocols outlined in Chapter 4: General materials and methods.

10.3.1.2 Preparation of colchicine and oryzalin solutions

Colchicine was prepared in a similar way to that for the seed treatments except that the stock solution was made at 10% and the concentrations tested were 0, 0.01, 0.1 and 1.0%. In order to increase cell permeability and absorption, 2% (v/v) of dimethyl sulfoxide (DMSO) was added. DMSO increases the permeability of cell walls and thereby improves the penetration of colchicine into the plant tissues, improving its action on the targeted cells (Hamill *et al.*, 1992).

Oryzalin stock was prepared by dissolving 0.1 g oryzalin into 10 mL DMSO giving the concentration of 1%. The solution was then diluted in distilled water to final concentrations of 0, 0.001, 0.0025 and 0.005% plus 2% (v/v) of DMSO.

10.3.1.3 The application of the solutions

Two-week old seedlings with fully developed cotyledons (Figure 70) were treated with colchicine or oryzalin. One drop of either colchicine or oryzalin was applied onto the apical meristem between the two cotyledons. Seedlings were kept in dark conditions for 24 hours to avoid the destructive effect of light on the colchicine or oryzalin. The humidity was kept as high as possible as drying increases the concentration and treated plants may die. Seedlings were then transferred back into the shaded glasshouse. Intermittent mist spray in the glasshouse was used to wash off the excess of the chemicals. Two weeks later, the seedlings were transferred to larger pots in a temperate glasshouse for further growth and development.

Figure 70.
Two-week old seedling in Jiffy™ pot treated with colchicine (arrow indicates the site of colchicine application). Bar = 1 cm.

The trials consisted of 10 replicates and were arranged in a Completely Randomised Design. An ANOVA was employed in data analysis and the Standard Errors (SEs) of means were calculated.

10.3.1.4 Observations

Leaf area and stomatal characteristics

Leaf area and stomatal characteristics (size and density) were used as initial parameters to assess the ploidy level of treated plants. Presumed tetraploids were examined for their chromosome numbers in the microspore mother cells in the same way as described in Chapter 5: Chromosome number.

Leaflet area was measured using a leaf area meter, model Delta-T. The instrument was calibrated with a known area of square black paper of 100 mm^2. As this observation used a destructive sampling method, only ten mature leaflets were taken from each replicate resulting in a total of 100 leaflets for each treatment. For uniformity of the treatment, all plants had entered the reproductive period and the third flower was fully opened. At this stage the leaflets had already reached their maximum size. In addition, all leaflets were taken from the fourth or fifth node on the central stem and the axillary branches growing from the base. The leaflets were sampled as shown on Figure 71. The area of each leaflet was measured by placing it on a tray. To flatten the leaflets, a clear glass

block (5 mm thickness) was placed over the top. The image was captured using a television camera (National WF-1400 E/A) and transferred to the leaf area meter; the numbers appearing on the monitor screen indicated the total area of the examined leaflet.

Mature leaflets were used for stomatal evaluations. For the uniformity, only the terminal leaflet was used (Figure 71). As this was a destructive sampling method, three leaflets were sampled from each plant. All leaflets were taken from leaves arising at the fourth or fifth node on the central stem and axillary branches. Samples to be evaluated were taken from the vein-free epidermal layer in the middle section of the abaxial side (lower surface) of the leaflet. To avoid variation due to environmental changes and to ensure the stomata were open to their maximum aperture, stomatal sampling was undertaken between 9.00 and 10.00 a.m.

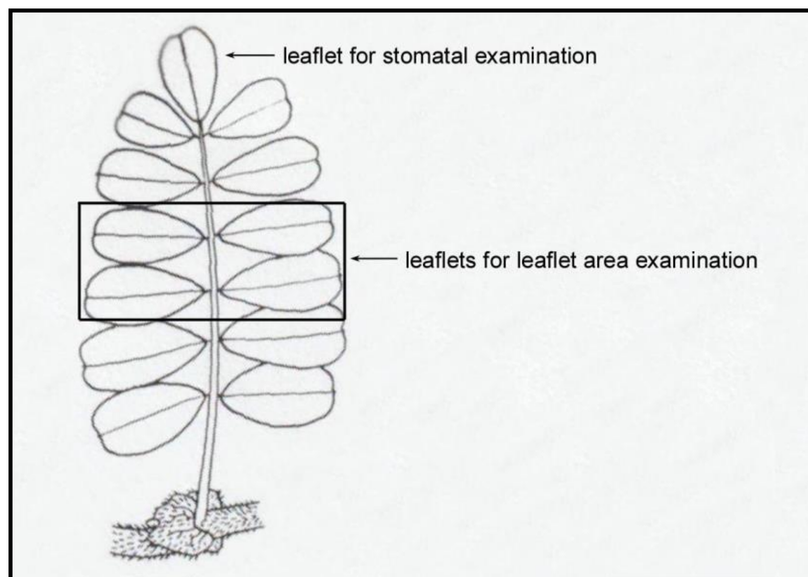

Figure 71.
Sampling of leaflets for the measurement of leaflet area and stomatal evaluation.

A thin layer of a clear nail polish was applied to the abaxial surface of the leaflets and left to dry. The nail polish was applied to the leaflets in the glasshouse while the leaflets were still attached to the plant. After several minutes, the leaflets were removed from the plant and placed between two

moistened filter papers in a closed Petri dish. The samples were kept at 4°C to keep them fresh before use on the same day.

By using forceps, the nail polish along with a layer of thin epidermis was peeled off and placed on a clean microscope slide. A drop of water was applied and a cover slip placed on the specimen. Observations were made with a light microscope at a magnification of 400x and the size and density of stomata were compared between non-treated and treated plants.

Only fully opened stomata were measured (Figure 72). The length of aperture was measured using a micrometer eyepiece where one division in the micrometer equal 2.5 µm under a magnification of 400x. Ten stomata from each leaflet were examined to obtain an average measurement.

For stomatal density, five places in the middle region of the abaxial surface of the leaflets were examined. The frequency of stomata was estimated under a magnification of 100x.

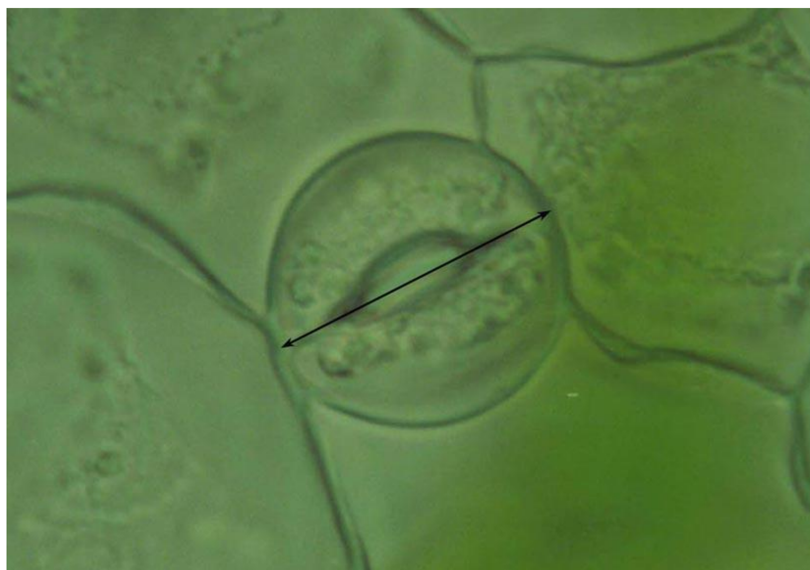

Figure 72.
The measurement of the length of stomatal aperture (as indicated by arrowheads).

Other characteristics

Having determined the tetraploid plants, the following parameters were examined: leaflet number per leaf, flower length, flag and keel widths, peduncle

length, pollen diameter and viability, and number of seeds per pod. These characteristics were assessed on those tetraploids that resulted from the treatments and on the control plants.

Total leaflet number per leaf was calculated when the plants had reached flowering. At this stage the plants had developed the maximum number of leaflets on every leaf. As the plants grew, the number of leaflets on the leaf after the first flower formation was liable to decrease. For uniformity, only those leaves located under the first inflorescence were used as samples. Five leaves, starting below the inflorescence, were sampled down each of central stem and similarly down each axillary branches. The average number of leaflets from these 15 leaves (obtained from 3 stems) was then calculated.

For the assessment of floral characteristics, ten inflorescences from each plant were sampled by taking 4 umbels from the central stem and 3 umbels from each of the axillary branches. The umbels were sampled from the first inflorescence upwards until the required number of umbels was obtained. All umbels were isolated from the plants one day after anthesis to ensure that the flowers have reached their maximum size. The measurement of flower length was made on graph paper, the flower being measured from the tip of the flag petal through to the tip of the keel petal (Figure 73a). The width of the flag petal was measured in a similar way to the flower length, with the widest part from left to right being measured on graph paper (Figure 73b). Similarly, the keel width of the same flower was also measured. Keel width measurement was taken on the widest part of the keel, approximately 2/3 of the distance from the tip of the keel (Figure 73c).

At the same time as the floral features were measured, the length of peduncle was measured from the junction with the stem through to the junction with the pedicels (Figure 74). The peduncle was cut from the plant and measured using graph paper.

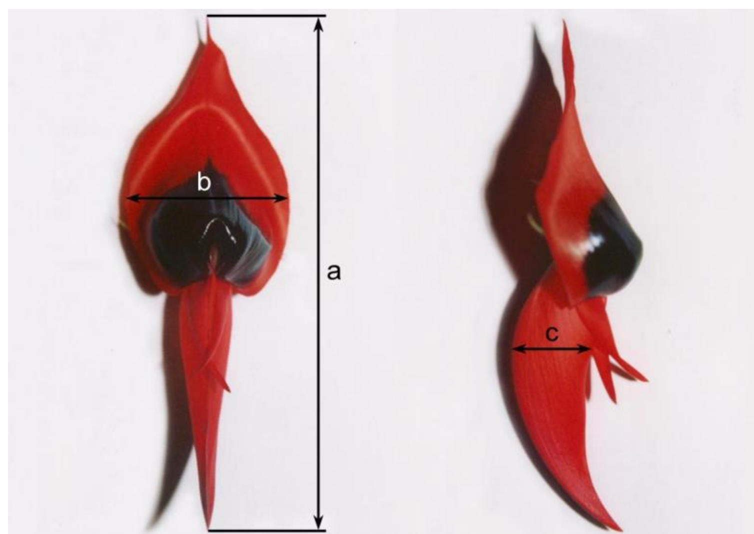

Figure 73.
Flower of *S. formosa* showing points for measurements. (a, measurements of flower length; b), measurement of flag width; c) measurement of keel width.

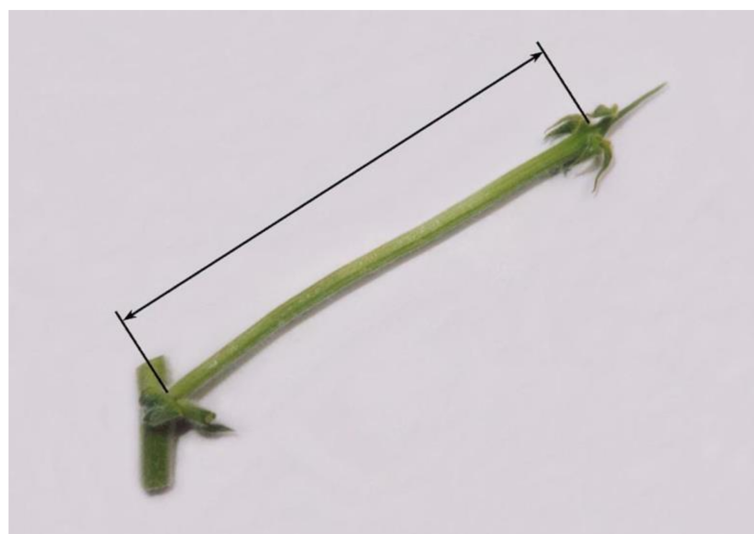

Figure 74.
Peduncle of flower of *S. formosa* showing measurement point.

Newly released pollen grains were collected from flowers at the time of anther dehiscence. Pollen grains were stained with one drop of 1% aceto-orcein on a microscope slide, covered with a cover glass (No. 1 thickness) and examined with a light microscope at a magnification of 400x. The diameter of viable pollen was measured (Figure 75) by using a micrometer eyepiece that gave 2.5 µm for each division at 400x. Pollen grains were sampled from five different

inflorescences (5 – 7 flowers) of each plant. In order to obtain a reliable measurement, ten pollen grains were measured for each flower. A total of 250 - 350 pollen grains were examined from each plant, depending on the number of flowers. The viability of pollen was assessed using the same techniques as described in Chapter 7: Pollen longevity and stigma receptivity, section 7.2.1.

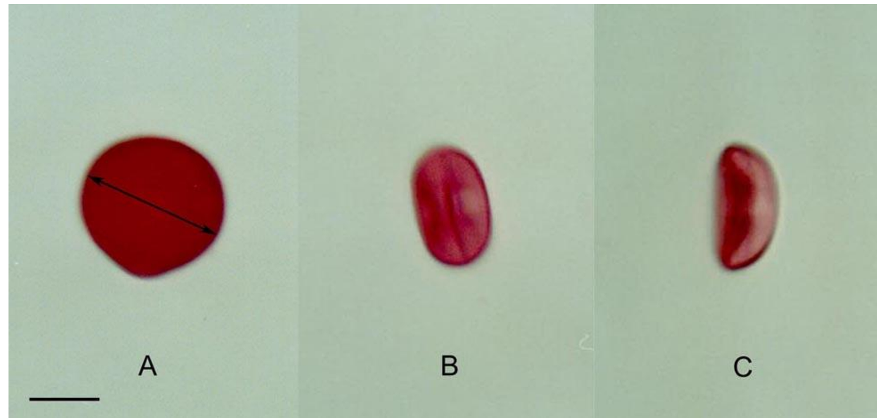

Figure 75.
Viable and unviable pollen grains of *S. formosa*. A, viable pollen grain that was used in determining pollen diameter as indicated by arrowheads; B and C are the examples of unviable pollen grains. Bar = 10 µm.

Both tetraploids and diploids were self-pollinated. All pods formed on each plant of each treatment were harvested and the number of seeds within each pod was counted. Only normal seeds were counted with abnormal seeds, which are smaller, thinner and whitish in colour, not included.

Flow cytometry (FCM) analysis

Seeds from treated plants were germinated and plants were allowed to grow to maturity in the glasshouse. The flow cytometry (FCM) technique was used to analyse ploidy levels of these plants. The flow cytometer model used was the FACS Vantage (Becton Dickinson, USA) (Figure 76) at The Commonwealth Scientific and Industrial Research Organisation (CSIRO) F.D. McMaster Laboratory, Chiswick, NSW. The instrument was calibrated with CaliBRITE™ Beads (Becton Dickinson, USA) before operation. The summary of the procedure is presented in Figure 77.

Figure 76.
Flow cytometer model FACS Vantage (source: CSIRO F.D. McMaster Laboratory, Chiswick NSW).

Five diploid and five tetraploid plants were analysed by FCM. The analysis was done as soon as possible after the samples were taken. Young leaflets were harvested and covered in wet filter paper in labelled Petri dishes to keep their freshness. Diploid *S. formosa* as an external standard was used for ploidy screening in this trial. All other samples were characterised by the relative position of their G1 peaks to the G1 peak of diploid *S. formosa.*

Approximately 50 mg of young leaflets were chopped up with a sharp and clean scalpel blade in a glass Petri dish. Intact interphase nuclei were released from the cut surface directly into 2 mL LB01 lysis buffer supplemented with live cell nucleic acid stain, Syto 13® (Molecular Probes, Eugene, Oregon USA). LB01 lysis buffer was prepared by mixing 363.4 mg Tris, 148.9 mg Na_2EDTA, 34.8 mg spermine tetrahydrochloride, 1.193 g KCl, 233.8 mg NaCl and 0.2 mL Triton X-100 in 200 mL distilled water at pH 7.5. A total of 0.22 mL β-mercaptoethanol was added to the mixture before being filtered through a 0.22 µm nylon filter. The mixture was stored at -10°C in 10 mL aliquots.

Approximately 2 mL of the homogenate-stained nuclei were filtered through a 45-µm nylon mesh into the analysis tube and its fluorescence was

analysed by FCM. The relative fluorescence intensity estimates the DNA content of more than 10000 nuclei of the samples at G1 phase.

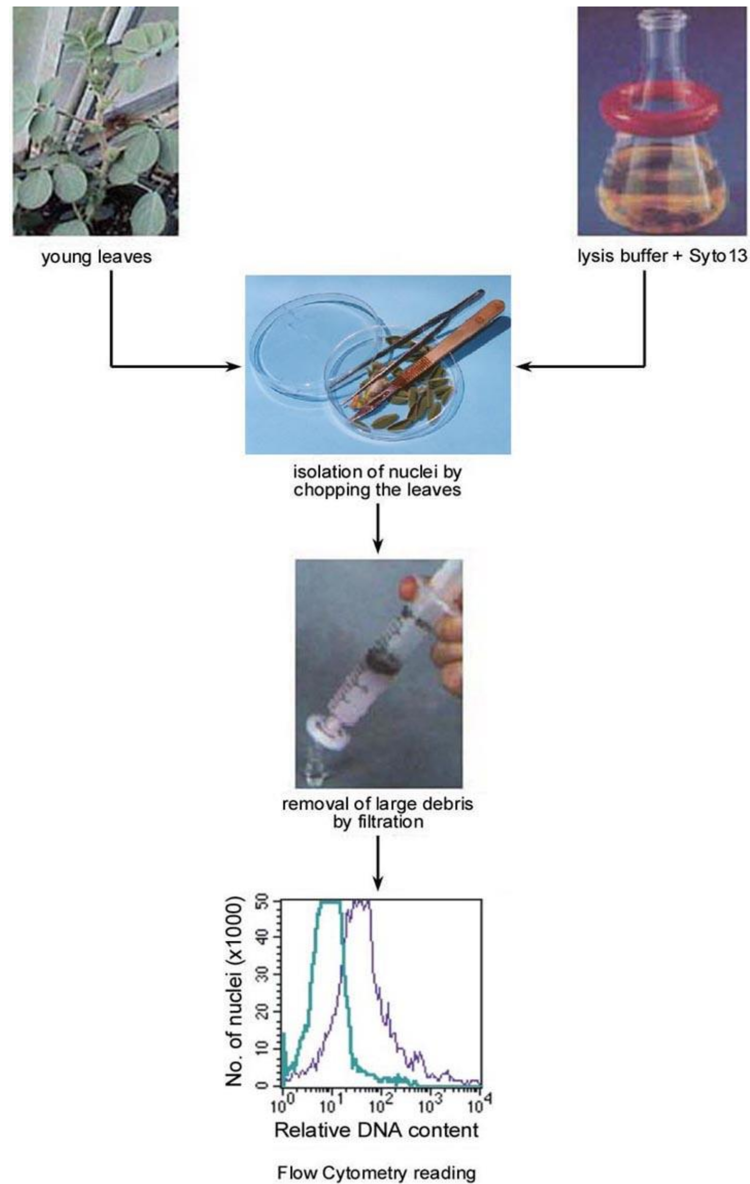

Figure 77.
Schematic diagram illustrating the protocol for determination of ploidy levels using FCM.

The reading produced in the form of a histogram was then set to channel 50 and used to determine the ploidy level of the sample as a standard reference for a diploid. In relation or comparison to the peak of the standard at channel 50, the G1 peak was different for tetraploids.

10.3.2 Results

10.3.2.1 Ploidy assessment in treated plants

Rate of conversion

The results showed that colchicine and oryzalin, when applied to seedlings, effectively induced tetraploidysation in *S. formosa*. The response, however, was selectively dependent upon the concentration of the treatment (Table 24). Higher frequencies of tetraploid induction were seen with 0.01 – 0.1% colchicine and 0.0025 – 0.005% oryzalin. Colchicine concentration higher than 0.1% was found to inhibit tetraploidysation and oryzalin concentration lower than 0.0025% was not effective. Colchicine application at 0.1% showed higher tetraploid induction than oryzalin at any concentration, and therefore colchicine was considered to be a more effective antimitotic agent in *S. formosa* compared to oryzalin.

Chromosome counts in the microspore mother cells, which showed $2n = 4x = 32$ (Figure 78), confirmed the determination of tetraploids using morphological indicators.

Table 24.
Effects of colchicine and oryzalin treatments on induction of tetraploids from seedling of *S. formosa.*

Treatments	No. of seedlings treated	No. of plants examined	% of plants with ploidy	
			Diploid	Tetraploid
Colchicine				
0.0 %	10	10	100	0
0.01%	10	10	60	40
0.1%	10	10	50	50
1.0%	10	10	70	30
Oryzalin				
0.0 %	10	10	100	0
0.001%	10	10	70	30
0.0025%	10	10	60	40
0.005%	10	10	60	40

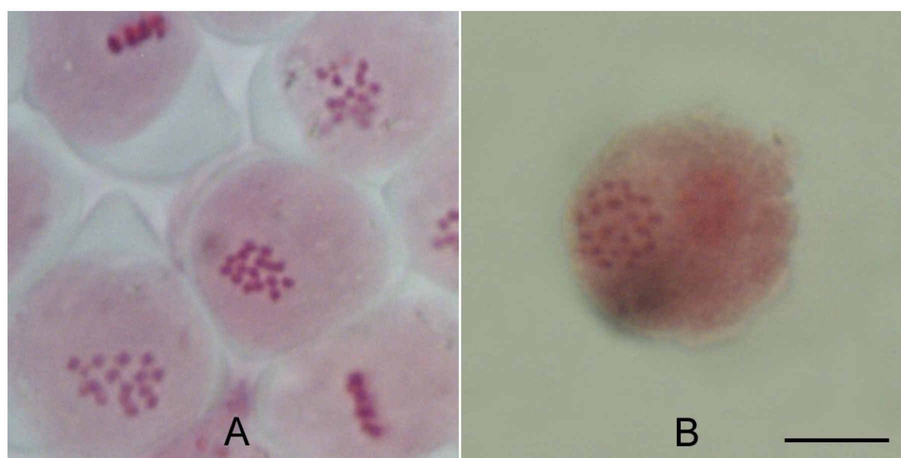

Figure 78.
Chromosomes in the microspore mother cells of *S. formosa*. A, diploid $2n = 2x = 16$; B, tetraploid $2n = 4x = 32$. Bar = 10 µm.

Leaflet area and leaflet number per leaf

The area of tetraploid leaflets was significantly larger than the leaflet of diploid leaves (both colchicine and oryzalin experiments produced $P < 0.01$). However, the number of leaflets per leaf was not different between tetraploids and diploids ($P = 0.77$ and $P = 0.50$ for colchicine and oryzalin experiments, respectively). Table 25 shows the comparison of leaflet area and leaflet number per leaf between diploids and tetraploids from both experiments.

Table 25.
Comparison of leaflet area and leaflet number per leaf between diploid and tetraploid *S. formosa.*

Ploidy level	Leaflet area	Leaflet number per leaf
Colchicine experiment		
Diploid	572.04 ± 37.91	12.27 ± 0.38
Tetraploid	1347.24 ± 34.61	12.11 ± 0.35
Oryzalin experiment		
Diploid	673.99 ± 35.41	12.13 ± 0.31
Tetraploid	1443.38 ± 33.76	12.41 ± 0.29

Comparing the tetraploid and diploid leaf, both had the rachis much the same length, but because the tetraploid leaflets were larger in area, they overlapped on the leaf (Figure 79A) compared to diploid leaflets (Figure 79B).

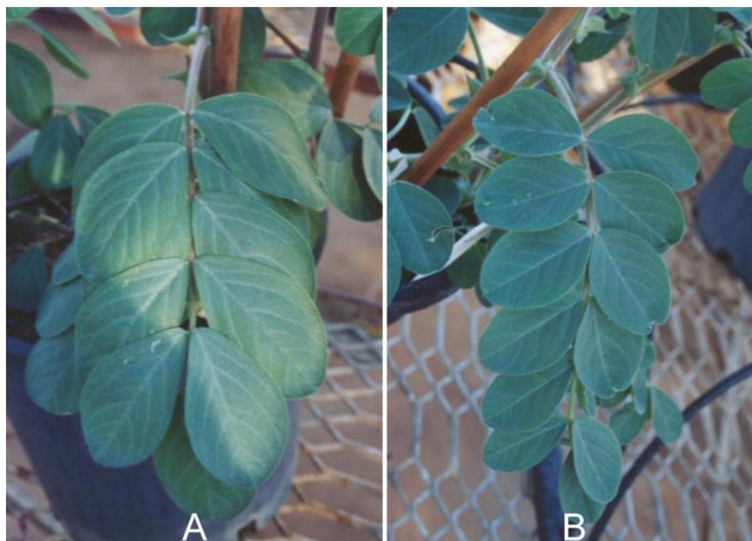

Figure 79.
Comparison of leaflets in tetraploid and diploid *S. formosa*. A, tetraploid; B, diploid.

This study showed that in addition to a significantly larger area, leaflets of tetraploids were also thicker than leaflets in diploids. Histological examination showed more spongy parenchyma in tetraploid leaflets than in the diploid leaflets (Figure 80), resulting in thicker leaflets. In addition to larger area and thicker leaflets, the tetraploid form of *S. formosa* had a stouter plant habit than the diploid (Figure 81A-B).

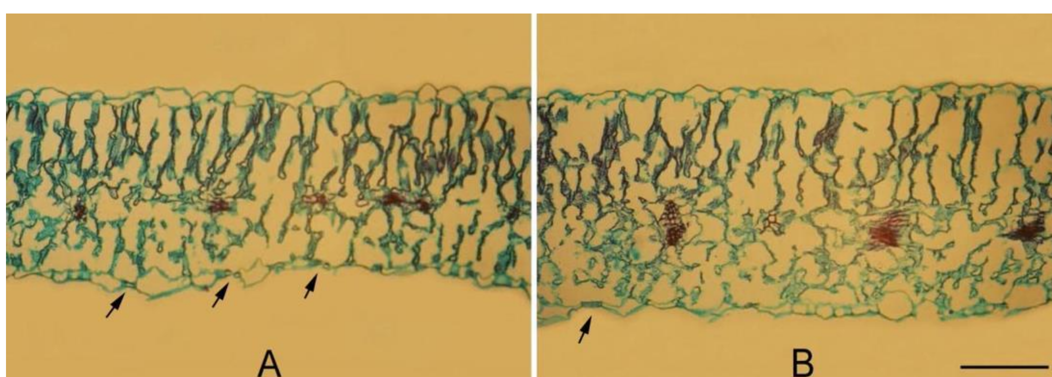

Figure 80.
Transverse sections of leaflets of diploid and tetraploid plants of *S. formosa* (arrows indicate stomata). A, diploid leaflet; B, tetraploid leaflet. Bar = 25 µm.

Figure 81.
Growth habits of diploid and tetraploid plants of *S. formosa*. A, diploid; B, tetraploid.

Stomatal characteristics

Subsequent analysis revealed that the induced tetraploids had a significantly lower stomatal density per unit area ($P < 0.01$) than diploids. In addition, stomatal size in the tetraploids was significantly larger ($P < 0.01$) than in the diploids (Table 26 and Figure 82).

Table 26.
Comparison of aperture length and stomatal density between leaflets of diploid and tetraploid *S. formosa*.

Ploidy level	Aperture length (µm)	Stomatal density per mm^2
Colchicine experiment		
Diploid	24.19 ± 0.46	116.29 ± 1.80
Tetraploid	36.54 ± 0.44	39.62 ± 1.72
Oryzalin experiment		
Diploid	23.45 ± 0.45	116.66 ± 2.10
Tetraploid	36.39 ± 0.43	38.49 ± 2.00

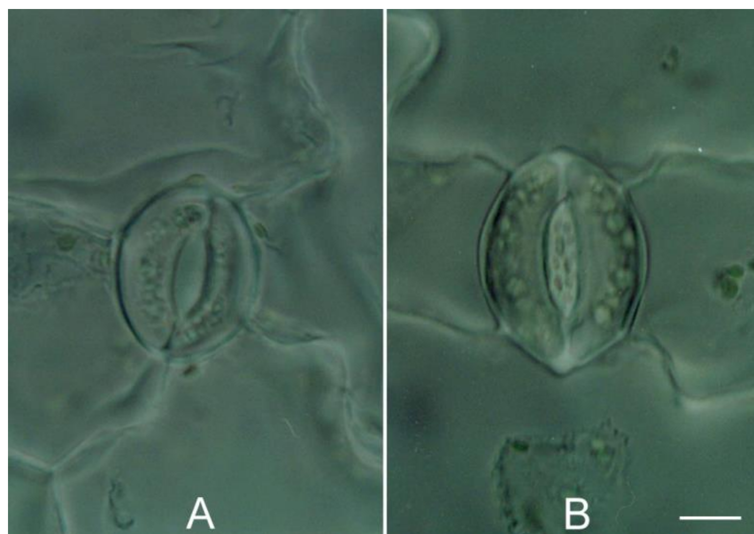

Figure 82.
Comparisons of stomata from diploid and tetraploid leaflets of *S. formosa*.
Bar = 10 μm.

Flower characteristics

The difference in flower length, keel width, flag width and peduncle length are shown in Table 27. The keel and flag width, as well as the peduncle length were larger in tetraploids than in diploids for both the colchicine and oryzalin experiments ($P < 0.01$). The flower length, however, did not show a significant difference between tetraploids and diploids ($P = 0.76$ and $P = 0.70$ for the colchicine and oryzalin experiments, respectively). In general it can be said that tetraploydisation effectively improved the flower quality of *S. formosa* as indicated by the wider flag and keel (Figure 83) as well as the longer peduncle, compared to the diploid flowers.

Table 27.
Comparisons of flower length, keel width, flag width and peduncle length between diploid and tetraploid *S. formosa.*

Ploidy level	Flower length (mm)	Keel width (mm)	Flag width (mm)	Peduncle length (mm)
Colchicine experiment				
Diploid	86.07 ± 0.27	32.37 ± 0.30	34.12 ± 0.32	122.30 ± 1.74
Tetraploid	85.96 ± 0.25	37.93 ± 0.27	42.54 ± 0.29	150.38 ± 1.58
Oryzalin experiment				
Diploid	85.98 ± 0.24	32.61 ± 0.13	32.78 ± 0.20	125.75 ± 1.24
Tetraploid	86.11 ± 0.22	37.95 ± 0.13	42.68 ± 0.19	150.14 ± 1.19

Figure 83.
Comparison of flowers from diploid and tetraploid plants of *S. formosa*. A, flower from diploid plant; B, flower from tetraploid plant.

Pollen size and viability and number of seed per pod

The colchicine and oryzalin experiments showed that the pollen diameter in tetraploids was larger than in diploids (Figure 84). However, the induced tetraploids had lower pollen viability. As a consequence, self-pollinated tetraploids produced fewer normal seeds per pod compared to diploids (Table 28).

Table 28.
Comparisons of pollen diameter and viability and number of seeds per pod between diploid and tetraploid *S. formosa.*

Ploidy level	Pollen diameter (µm)	Pollen viability (%)	Normal seeds per pod (%)
Colchicine experiment			
Diploid	21.27 ± 0.22	65.30 ± 0.83	98.87 ± 1.37
Tetraploid	27.72 ± 0.20	47.35 ± 0.76	53.93 ± 1.25
Oryzalin experiment			
Diploid	21.48 ± 0.24	65.16 ± 0.67	98.95 ± 0.52
Tetraploid	28.45 ± 0.23	47.51 ± 0.64	53.91 ± 0.50

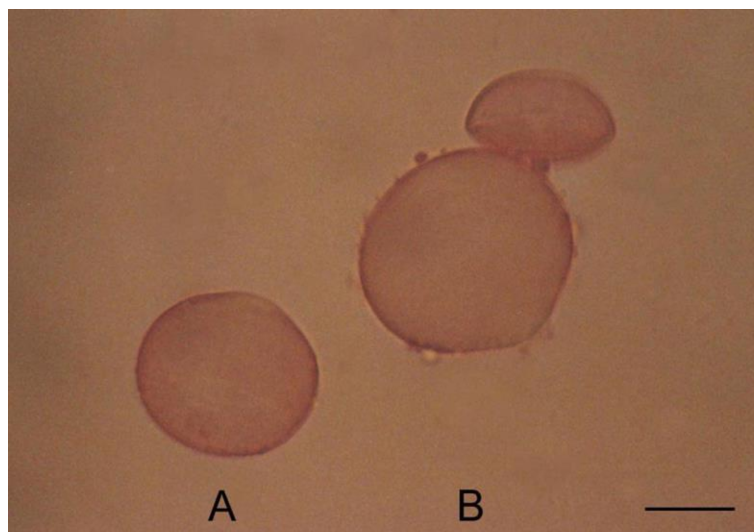

Figure 84.
Comparison of pollen size from diploid and tetraploid plants of *S. formosa*. Bar = 10 µm.

10.3.2.2 Ploidy assessment in regenerated plants

Although the treated plants examined in the previous experiment showed the induction of tetraploidy, their regenerants did not show tetraploid characteristics. This was shown by the histograms, which resulted from the flow cytometry analysis showing no difference in the G1 peak between the regenerants of treated plants and the normal plants (Figure 85). In addition, visual observations on plant morphology and growth habit did not show any differences between the two progenies.

10.4 Crossing F1 of treated plants with normal plants

10.4.1 Materials and methods

The F1 regenerants of each colchicine and oryzalin treatment were crossed with normal (untreated) plants. Cross-pollination was made in two ways, firstly with the normal plant as the source of the female parent and secondly with the normal plant as the source of the male parent. Flowers were emasculated one day before anther dehiscence and pollinated two days later when the stigma was most receptive (see Chapter 7: Pollen longevity and stigma receptivity). Pollination was carried out manually by rubbing the stigma several times with a fingertip

covered with pollen grains. Seeds resulting from this crossing were germinated and the plants were grown in the glasshouse for evaluation.

10.4.2 Results

All seeds germinated well and the plants grew to flowering. However, as with experiment mentioned earlier (Seedling treatment with colchicine and oryzalin, section 10.3.2.2), all plants showed normal (diploid) characters. The flow cytometry analysis showed no difference in the G1 peaks of the two progenies tested (Figure 85). Furthermore, all plants produced pollen grains, and pods were formed after self-pollination, as in normal plants.

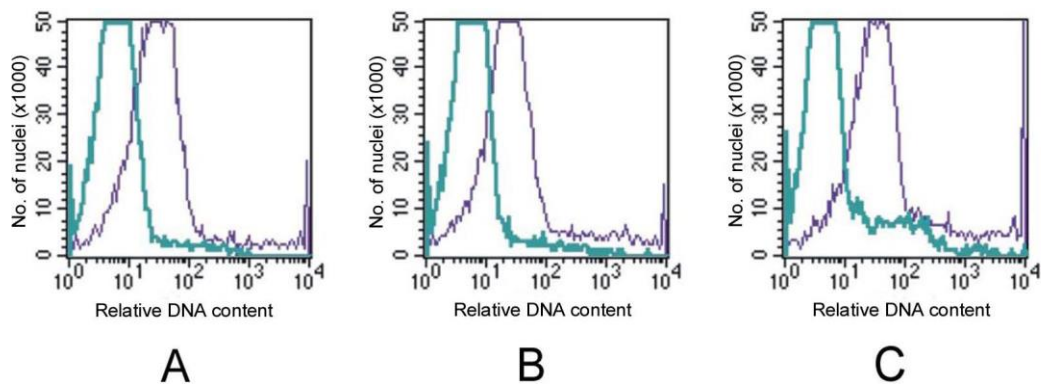

Figure 85.
Histograms of flow cytometry analysis on the ploidy level of *S. formosa*. A, control plants; B, F1 regenerants of treated-plants; C, the crossing of A × B.

10.5 Discussion

Colchicine and oryzalin have been successfully used to induce polyploidy in many plants (Griesbach, 1990; Varhoeven *et al.*, 1990; Hamill *et al.*, 1992; Smith and Hamill, 1996; Takamura and Miyajima, 1996; Takamura *et al.*, 2002). This study showed that seedling treatment with either colchicine or oryzalin proved effective in the induction of tetraploidysation in *S. formosa*. However, since the conversion rate is very low, further studies will be needed to find more effective methods of tetraploid induction in this species. On the other hand, seed treatment with colchicine was found ineffective and is liable to result in abnormal seed germination (Figure 69).

The detrimental effect of colchicine on seed germination may be caused by the concentrations tested, which were too high. A similar effect of colchicine on seed germination is also reported by Takamura and Miyajima (1996) in *Cyclamen persicum.* In addition, treating the whole seed undergoing germination will affect all parts of the seedling, although the only target and the only part that must be affected is the growing point. The major weakness of the method is that the roots are affected and usually the growth of affected roots is either poor or inhibited. Meanwhile, lower concentrations of colchicine were not effective in inducing polyploidysation in *S. formosa* due to the hard seed coat limiting the access of colchicine to the growing point. The embryological study (see Chapter 6: Embryological development and sexual reproduction) showed that the seed coat of *S. formosa* consisted of thick-walled columnar palisade cells on the outside and a mesophyll with a layer of hypodermal osteosclereids. This structure is believed to restrict the effectiveness of colchicine at lower concentrations.

Significant morphological differences exist between diploid and tetraploid *S. formosa.* In general, tetraploid plants are bigger and stouter than diploid plants (Figure 81). This is similar to the observation of Griesbach (1990) on tetraploid *Anigozanthos* hybrid produced by colchicine treatment. Furthermore, the status of ploidy level of *S. formosa* correlates positively with stomatal size; the higher the chromosome numbers the larger the stomatal size. Meanwhile, a negative correlation is found with stomata density; the higher the chromosome numbers the fewer stomata per unit area. Therefore, stomatal size and density are useful indicators to distinguish diploids from tetraploids resulting from colchicine and oryzalin treatments. Proportional relationships between ploidy level and stomatal size and density are also found in *Bromus inermis* (Tan and Dunn, 1973), *Hordeum vulgare* (Borrino and Powell, 1988), *C. persicum* (Takamura and Miyajima, 1996) and *Musa* spp. (Hamill *et al.*, 1992; Azhar *et al.*, 2002). Stomatal characteristics have been used effectively to distinguish tetraploids from diploids in these species.

A positive relationship exists between pollen diameter and ploidy level in *S. formosa.* As with *B. inermis* (Tan and Dunn, 1973), *Musa* spp. (Tenkouano *et al.*,

1998) and *C. persicum* (Takamura and Miyajima, 1996) tetraploidysation of *S. formosa* results in larger pollen size than pollen in diploid plants. However, the viability of tetraploid pollen is significantly lower than that of diploid pollen. The results of this research confirmed the negative relationship between ploidy level and pollen viability as reported by Takamura and Miyajima (1996) in *C. persicum*.

The differences between tetraploids and diploid in flower size are shown in Table 27. The flower lengths of the tetraploids were not significantly different from those of the diploids. However, the widths of the keel and flag petals in tetraploids were larger than in diploids. Similarly, Takamura and Miyajima (1996) reported that the petal size in tetraploid *C. persicum* was larger than in diploid plants. Larger flower size was also reported by Griesbach (1990) on tetraploid *Anigozanthos* hybrid produced by colchicine treatment.

This experiment showed that the response of *S. formosa* to colchicine and oryzalin treatment is reversible. Plants treated with colchicine and oryzalin exhibited tetraploid characteristics (previously described) but their F1 progeny seemed to lose those characteristics and reverted back to their diploid nature. Ploidy analysis using flow cytometry showed no difference in the pattern of the histograms that resulted from diploid and tetraploid-derived plants. Further examination on the chromosome numbers confirmed that the progeny was diploid. As a consequence, the crossing of the F1 of treated plants with normal plants did not result in triploids as previously expected. The reversion of ploidy status was also reported by Hamill *et al.* (1992) on tetraploid banana induced by colchicine treatment under *in vitro* conditions. They found that reversion to the diploid level was encountered during micropropagation of colchicine-treated plants as well as after field establishment.

10.6 Conclusion

The techniques reported here produced tetraploids quickly and simply, and should find applications in the breeding programmes of *S. formosa.* However, while polyploidysation using colchicine and oryzalin represents a significant

improvement in the breeding of diploid *S. formosa* in terms of flower quality, the reversion to diploid by F1 progeny is a significant impediment to the production of large-scale tetraploids, as well as the production of triploid hybrids. Further study is recommended to investigate effective methods of polyploidysation that will produce a high rate of tetraploid conversion and, most importantly, also produce persistent stability in changes in ploidy level. Stable tetraploid progeny are undoubtedly important for the steady production of triploid *S. formosa.* Screening from a much larger population may increase the chance of more stable plants.

CHAPTER 11
GENERAL DISCUSSION AND FUTURE DIRECTIONS

Petal staining by the pollen grains released by dehiscing anthers and quick degeneration of pollinated flowers resulting in poor flower quality are the major impediments to the commercialisation of *Swainsona formosa* as a cut flower. Therefore, the main objective of this work was to develop a breeding programme to improve the quality of *S. formosa* flowers for better market value. Techniques to produce male-sterile flowers were investigated via *in vivo* strategies using polyploidy-inducing chemicals and by *in vitro* strategies involving microspore embryogenesis using anther culture. Since *in vivo* and *in vitro* methods were targeted at producing plants with modified ploidy levels, the identification of chromosome number in diploid *S. formosa* was a necessary prerequisite step to determine whether the strategies were successful.

Many authors reported that chromosome number in the genus *Swainsona* was $2n = 2x = 32$ (Hair, 1963; Ermayanti *et al.*, 1993; Thompson, 1993; Heenan, 1998b; Buza *et al.*, 2000). However, Sands (1975) found that the chromosome number in *S. galegifolia* was $2n = 2x = 16$. Tade (1992) investigated the diploid chromosome number of *S. formosa* and assumed it was $2n = 2x = 12$. This study revealed that the chromosome number in *S. formosa* is $2n = 2x = 16$. This finding confirmed the base chromosome number of $x = 8$ in the tribe Gelegeae as previously proposed by Goldblatt (1991). Results were obtained from a population of plants grown in the glasshouse over three years. This indicates that *S. formosa* has a stable chromosome number under controlled growing conditions. It is therefore suggested that future work should be focussed on populations obtained from the field. An investigation of chromosome number in *S. formosa* from different sites throughout Australia would be a tremendous contribution to the botany and systematics of this species.

As in most members of the family, the flowers of *S. formosa* consist of five sepals and a five petals (2 of which are fused to form the keel). There are ten stamens, of which nine are fused by their filaments, and one posterior stamen that

remains free. The stigma is covered by a stigmatic cuticle, which is characteristic of most papilionoid legumes (Arroyo, 1981). The anther is tetrasporangiate with a single cell layers of endothecium, middle layer and tapetum below the epidermis. The development of the anther wall conforms to the dicotyledonous type. Cytokinesis in microsporocytes occurs simultaneously resulting in tetrahedral microspore tetrads. Mature pollen grains are triporate and shed at the 2-cell stage. Mature ovules are campylotropous with the micropyle in a zigzag pattern. Polyembryony has never been reported in the genus so far but in the this study more than one embryo sac were found in some ovules. The endosperm development is of nuclear type but it becomes cellular at a later stage and is mostly consumed by the embryo.

The seed coat of *S. formosa* is composed of a layer of palisade cells and a layer of hypodermal sclereids. This type of organisation and development of the seed coat is typical of the legume family (Corner, 1951) except in *Psophocarpus tetragonolobus* (Lim and Prakash, 1997), in which the hypodermal sclereid layer is absent from the seed coat. It is worthwhile to carry out further investigations to obtain a more detailed picture of the embryological development of *S. formosa.*

In conventional plant breeding, creating particular traits through cross-pollination in the glasshouse or in the field has been a popular method of producing hybrids. The most effective way involves hand-pollination by manually pollinating the stigma with pollen from other individuals by means of fingertips or using a paintbrush. Therefore, it is important to ensure the availability of highly viable pollen grains at any stage of the breeding programme by keeping pollen under certain storage conditions. Experiments on pollen longevity showed that pollen left within the keel of the flowers in the glasshouse only retained their viability for up to 4 days after dehiscing. Storing pollen at 4°C and in total darkness prolonged the longevity for up to 28 days after dehiscing. Longevity was even better if pollen grains were kept at -10°C under total darkness; pollen viability remained high for the duration of experiment (two months after dehiscing). This indicated that storage of *S. formosa* pollen at 4°C or at -10°C wa sufficient to maintain high viability. Although pollen of *S. formosa*

may survive to about -180ºC (Hughes *et al.*, 1991) such a method requires special techniques and facilities. This study provided a simple and economically sound protocol for preservation of *S. formosa* pollen for breeding purposes. It is suggested that future investigations should be directed to test the germinability of pollen grains on the stigmatic surface in intact plants after a certain period of storage at either 4ºC or -10ºC.

Stigmas were receptive from one day before anther dehiscence and the receptivity reached its peak within 4 days after anther dehiscence. However, although pollen viability and stigma receptivity overlapped, the presence of stigmatic cuticle as reported by Arroyo (1981) and Jusaitis (1994) often became a limiting factor in self pollination.

The *in vitro* strategy to produce haploid plants using anther culture techniques has been discussed in Chapter 8. The first important step was to determine the correct stage of microspore development using floral bud size as a morphological predictor. The result of this reseach suggests that the length of floral buds can be used as a reliable tool for the prediction of the microspore developmental stage in *S. formosa*.

The attempt to induce microspore embryogenesis was preceeded an examination of the effect of microspore developmental stage, media types, light spectra, pre-treatment of anthers prior to culture, plant growth regulators and the addition of Ficoll to the medium on the response of anthers in culture. The results of the experiments indicated that the response of anthers in culture is greatly influenced by anther pre-treatment, plant growth regulators and the addition of Ficoll to the culture medium. Anther pre-treatment with mannitol starvation at 4ºC was found to increase callus formation although it may be insufficient to induce androgenesis. In some species stress pre-treatment was necessary to induce microspore embryogenesis (Kyo and Harada, 1986; Touraev *et al.*, 1996; Höfer *et al.*, 1999) but in recalcitrant plants such as legume species (Taji et al., 2002) it would be necessary to combine stress pre-treatment with other factors (Immonen and Robinson, 2000). Therefore, future work needs to focus on

investigating the effect of mannitol starvation at 4°C in combination with various cultural conditions.

The presence of auxins and cytokinins in the culture medium was found to increase callus formation in *S. formosa* anthers. However, the response of the cultured anthers was not specific. Callus cultures were initiated using callus from anthers cultured on media supplemented with IBA+kinetin and IBA+zeatin but subsequent development was limited. The major achievement was the regeneration of shoots and roots, however, the shoots and roots were found to be hyperhydrated and failed to develop further in the new medium as they became necrotic and chlorotic, and eventually died.

Ficoll was used to improve the response of the anthers in experiments on anther culture (Chapter 8). Isolated anthers pre-treated with mannitol starvation at 4°C showed an improved callusing capacity when cultured on a double-phase medium supplemented with 49.3 µM IBA + 4.61 µM zeatin and 10% Ficoll. Embryogenic efficiency of anther-derived callus was also improved (Table 18). Somatic embryos were induced to develop on a growth regulator-free medium but hyperhydration and fasciation became the limiting factors. The addition of Ficoll was found also to improve shoot and root differentiation (Table 19). However, again hyperhydration led to the failure of the embryos and shoots to develop further.

Hyperhydration is a major problem in tissue culture of *S. formosa* (Taji and Williams, 1989; Tade, 1992; Tapingkae, 1998; Sudhersan and AboEl-Nil, 2002). The cause of hyperhydration is probably attributable to the excess of cytokinin levels in the culture medium, low matrix potential and the accumulation of ethylene concentration in culture vessels (Kevers *et al.*, 1984). A high level of ammonium and water content in the gaseous phase in the culture flask was also found to cause hyperhydration (Rice *et al.*, 1992). In this context, it would be worthwhile if future research concentrated on restoring hyperhydrated *S. formosa* shoots to normality.

Although callus formation was promoted by pre-treatment of anthers prior to culture, the application of plant growth regulators and the use of Ficoll in a

double-phase medium, histological examination revealed that the callus did not proliferate from the microspores. Callus did not proliferate from the epidermis of the anther wall either. Instead, callus grew from the endothecium, middle layer or tapetum cells. Although the objective to induce androgenesis in *S. formosa* was not accomplished, these experiments showed that the explants had the capacity to regenerate plants. Further study on the development of *S. formosa* microspores under *in vitro* condition is needed to reveal the factors limiting microspore embryogenesis. Bayliss *et al.* (2002) reported that the strong exine layer had become the barrier for further development of pro-embryos in microspore culture of *Lupinus* spp. If *S. formosa* is considered to be similar, then future work should be focussed on microspore culture investigating chemical or physical or the combination of chemical and physical methods to overcome the barriers preventing microspore embryogenesis. Perhaps storing microspores at low temperatures (-10°C) prior to culture may overcome the barriers (see Chapter 7: Pollen longevity and stigma receptivity).

The vegetative stage has been recognised as the best stage for the induction of polyploidy in many plants (Lucket, 1989; Takamura and Miyajima, 1996; Azhar *et al.*, 2002; McCuistion and Wehner, 2002; Takamura *et al.*, 2002). Once the plants have entered their reproductive stage the induction of polyploidysation is no longer effective. This study showed that the period between the 56th and 60th day after germination was the critical period for the conversion from vegetative to reproductive growth in *S. formosa* grown under a 12- to 16-hour photoperiod in the glasshouse. This suggested that any treatment to induce polyploidysation should be done no later than 56 days after seed germination. Another simple way of determining this conversion time is by using the number of nodes before the first flower as a morphological indicator. This study revealed that the 10th and 8th nodes on the central stem and axillary branches, respectively, were the critical nodes for the conversion from vegetative to reproductive growth.

In addition, plants grown under 16-, 12- and 8-hour photoperiods in growth cabinets produced more nodes before the first flower initiation, and the 12th and 11th nodes are considered as the critical nodes for the central stem and axillary

branches, respectively. Although the plants grown under the 8-hour photoperiod showed poorer growth habits and took longer to flower (Yusuf *et al.*, 2002), no significant difference in the number of nodes before the first flower was noted among plants grown under the three different photoperiod regimes. This finding suggests that the node to first flower may be genetically determined in *S. formosa* and unaffected by photoperiod, and thus can be effectively used as a morphological predictor for first flower initiation for plants grown in a growth cabinet. Future work is suggested to focus on the influence of other seasonal factors such as light intensity and temperature to obtain a complete picture of the conversion from vegetative to reproductive growth under different seasonal variations.

An improvement in the quality of economically important parts of plant such as flowers (Griesbach, 1990; Takamura and Miyajima, 1996) or rhizomes (Smith and Hamill, 1996) as the result of tetraploid induction using antimitotic chemicals has made the use of colchicine and oryzalin increasingly popular these days. The same technique was also applied to *S. formosa* and the results have been discussed in Chapter 10. It was shown that either colchicine or oryzalin can effectively induce polyploidysation in *S. formosa*. However, the response seems to be transient as the regenerants (F1) of treated plants reverted to diploidy when grown in the glasshouse. Reversible polyploidysation was also reported by Hamill *et al.* (1992) on autotetraploid *Musa* spp. obtained from micropropagation. The reversion to the diploid status in *S. formosa* may be due to the recalcitrance of legume tissues. As a consequence, the efforts of producing triploids using the methods developed in this study was unsuccessful.

Future work in polyploidy induction in *S. formosa* needs to look at other methods such as the use of gamma irradiation and temperature shock. The *in vitro* application of colchicine or oryzalin is another recommendation for future work on the production of autotetraploid *S. formosa.* This *in vitro* technique of chromosome doubling has been reported to be effective in *Anigozanthos* hybrid (Griesbach, 1990), *Gerberra jamesonii* (Honkanen *et al.*, 1991), *Cyclamen persicum* (Takamura and Miyajima, 1996) and *Lilium longiflorum* (Arzate-

Fernández *et al.*, 1997; Takamura *et al.*, 2002). However, it should be noted that colchicine is very harmful to human beings and is suspected to be carcinogenic; it should be handled and disposed of with extra care. Meanwhile, oryzalin is reported to be much safer than colchicine but it is expensive and is not available for commercial use. Takamura *et al.* (2002) reported Surflan™ (Surflan) as another chemical that has the ability to induce polyploidysation in plants. Surflan contains 40% oryzalin as an active ingredient and relatively cheaper in price. Therefore, it can be used as an alternative for colchicine and oryzalin to induce safe and economical polyploidysation in *S. formosa*.

Another possible way to induce male-sterility, in addition to crossing tetraploid with diploid, is to use molecular techniques and introduce a "male-sterility gene". Yamamoto *et al.* (1997) found that male-sterility in citrus was controlled by the interaction between nuclear and cytoplasmic genes, resulting in gene-cytoplasmic male-sterility. Isolation of a male-sterility gene and introducing it in transformation could result in male-sterile *S. formosa.*

In conclusion, although the main objective was not fully accomplished this study has resulted in valuable information on the fundamental aspects of breeding strategies in *S. formosa.* New reports on the chromosome number, embryological development and sexual reproduction, pollen longevity and stigma receptivity, the seasonal influence on floral apex development, problems and limitations in the induction of microspore embryogenesis and polyploid induction have been revealed. Any future research needs to focus on overcoming any limitations or problems noted in this research, using alternatives as proposed within the research.

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APPENDIX 1
PUBLICATIONS ARISING FROM THE STUDIES REPORTED IN THIS THESIS

Refereed Papers

1. Chromosome number in *Swainsona formosa* (Fabaceae). (New Zealand Journal of Botany, 2002, Vol. 40:331-333)
2. Towards sterile plant production in Sturt's desert pea (*Swainsona formosa*) via anther culture, pp. 145-157. In 'The Importance of Plant Tissue Culture and Biotechnology in Plant Sciences' (Eds A. Taji and R. Williams). Proceeding of the 7th Meeting of the International Association for Plant Tissue Culture & Biotecnology (The Australian Region), Armidale, NSW, 20th – 23rd January 2002. University of New England, Armidale.
3. Towards sterile plant production in Sturt's desert pea (*Swainsona formosa*) via *in vivo* triploid plant production, pp. 377-383. In 'The Importance of Plant Tissue Culture and Biotechnology in Plant Sciences' (Eds A. Taji and R. Williams). Proceeding of the 7th Meeting of the International Association for Plant Tissue Culture & Biotecnology (The Australian Region), Armidale, NSW, 20th – 23rd January 2002. University of New England, Armidale.
4. *In vitro* callus induction and differentiation in Sturt's desert pea (*Swainsona formosa*), pp. 113-118. In 'Plant Breeding for The 11th Millennium' (Ed. J. A. McComb). Proceeding of the 12th Australasian Plant Breeding Conference, Perth, Western Australia, 15th – 20th September 2002. The Australasian Plant Breeding Association Inc., Perth.
5. Determining microspore developmental stage as the first step in Sturt's desert pea anther culture, pp. 261-264. In 'Plant Breeding for The 11th Millennium' (Ed. J. A. McComb). Proceeding of the 12th Australasian Plant Breeding Conference, Perth, Western Australia, 15th – 20th September 2002. The Australasian Plant Breeding Association Inc., Perth.
6. Pollen viability and stigma receptivity in Sturt's desert pea, pp. 265-268. In 'Plant Breeding for The 11th Millennium' (Ed. J. A. McComb). Proceeding of the 12th Australasian Plant Breeding Conference, Perth, Western Australia, 15th – 20th September 2002. The Australasian Plant Breeding Association Inc., Perth.

Published Abstracts

1. Screening auxins and cytokinins in Sturt's desert pea (*Swainsona formosa*) anther culture. (4th International Legume Conference, Canberra, 2nd – 6th July 2001).

2. Breeding strategies used in production of sterile Sturt's desert pea (*Swainsona formosa*) flowers. (20th International EUCARPIA Symposium Section Ornamentals: Strategies for New Ornamentals, Melle, Belgium 3rd – 6th July 2001).

APPENDIX 2
ABBREVIATIONS AND TERMS USED IN THIS THESIS

2,4-D = 2,4-dichlorophenoxyacetic acid

2iP = 2-isopentenyladenine

ABA = abscisic acid

BAP/BA = 6-benzylaminopurine/benzyladenine

DMSO = dimethyl sulfoxide

EDTA = ethylenediamine tetraacetic acid

FPA = formalin-propiono-alcohol

IAA = indole-3-acetic acid

IBA = indole-3-butyric acid

MS = Murashige and Skoog (1962)

NAA = naphthaleneacetic acid

PVP = polyvinylpyrrolidone

STS = silver thiosulphate

TBA = tertiary butyl alcohol

TIBA = 2,3,5 triiodobenzoic acid

TDZ = thidiazuron