Accelerating the generation of homozygosity and genome fixation in pea (*Pisum sativum* L.)

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Abstract

Pea cultivars are nearly homozygous and thus homogeneous when they are released. The traditional method of selfing is slow and inefficient, taking up to ten generations of inbreeding following a cross to achieve a high level of homozygosity. Current single-seed-descent (SSD) methodologies enable a maximum of three generations per year to be developed in pea. Doubled haploidy and an in vitro based modified SSD technology have been utilised in many important crops for the rapid achievement of homozygosity, and thus acceleration of the breeding process. In pea, due to the lack of robust protocols, neither of these technologies is routinely used in a breeding program. The aim of this study was to accelerate the breeding process in pea by developing in vitro techniques to more rapidly achieve a high level of homozygosity and to gain a better understanding of the fundamental mechanisms involved in these processes. These techniques include: 1) haploidisation from cultured anthers in selected genotypes of varying backgrounds; and 2) in vitro flowering and seed-set for use in SSD breeding strategies. The development of robust genotype-independent in vitro protocols will be of great value to accelerate the breeding process in pea.

In this research a number of key factors in the development of a robust pea anther culture protocol were identified and optimised. The combined application of multiple stress treatments, including the novel stress agent sonication, and the optimisation of key culture factors led to the development of an efficient protocol for the routine induction of androgenesis and embryo production from extracted anthers of various pea genotypes. A flow cytometry study was undertaken to further understand the effect of individual and combined stress treatments on androgenesis elicitation. Analysis of the flow cytometry results revealed clear differences in the relative nuclear DNA content of microspores within anthers after stress treatments
and enabled prediction of whether a combination of stresses were elicitors or enhancers of androgenesis. Flow cytometry is thus proposed as a method for the quick assessment of the effect of individual and combined stress treatments, based on the relative nuclear DNA content.

An optimised *in vitro* based SSD system was developed which enabled rapid *in vitro* flowering and seed-set across a range of pea genotypes including, for the first time, mid to late flowering types. In this protocol, the antigibberellin Flurprimidol was used to control *in vitro* plant size, and plants with the meristem removed and excised shoot tip explants were cultured into glass tubes under white fluorescent light. The involvement of antigibberellin and light quality on plant growth response is discussed. Using this strategy more than five generations per year can be obtained with mid to late flowering genotypes and over six generations per year for early to mid flowering genotypes.

The results presented in this research form a solid basis for further efforts designed to enhance androgenic response in pea and extend double haploid technology to other legumes. However, further research is required before this technology will be routinely available within a pea breeding program. In the absence of a robust DH protocol, the *in vitro* based SSD system reported herein will offer a valuable alternative method for the rapid achievement of homozygosity by shortening each generation cycle.
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**Statement of candidate contribution**

This thesis contains no material which has been submitted for the award of any other degree or diploma in any other University and to the best of my knowledge it contains no copy or paraphrase of material previously published by any other person, except where due reference is made. All the glasshouse and laboratory work related to this thesis was carried out solely by Federico Ribalta. The publications pertaining to this thesis were written by Federico Ribalta and the co-authors were involved in the experimental planning phase, discussion of results, structure of the papers and editorial comments to finalise them.
Publications pertaining to this thesis

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**Abbreviations:** ABA - abscisic acid; BAP - 6-benzylaminopurine; DAPI - 4',6-diamidino-2-phenylindole dihydrochloride; DH - doubled haploid; FDA - fluorescein diacetate; GA - gibberellic acid; GFP - green fluorescent protein; GMO - genetically modified organism; HSP - heat shock protein; IAA-asp - indol-3 acetic acid-asparagine; MS - Murashige & Skoog; NAA - a-naphthalene acetic acid; RIL - recombinant inbred lines; SSD - single seed descent; 2,4-D - 2,4-dichlorophenoxyacetic acid.
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Introduction and literature review

1.1 Introduction

Pea (*Pisum sativum* L.) is one of the world’s oldest domesticated crops. It is the third most widely grown legume, as its seeds serve as a protein-rich food for humans and livestock alike (Smýkal et al., 2011). It is also important in dry-land cropping systems both as a cash crop and for the provision of soil nitrogen from the fixation of atmospheric nitrogen (Redden et al., 2005). Peas are a useful component in cropping rotations, providing overall economic value in the cropping system (Muehlbauer, 1996).

Breeding against diseases, insect pests and abiotic factors has played a significant role in improving yields in pea (Khan and Croser, 2004); however, these breeding objectives have been constrained by the absence of biotechnology tools applicable to the crop (Croser et al., 2006; Smýkal et al., 2012). Pea cultivars are nearly homozygous and homogeneous when they are released. The traditional method of selfing is very slow and inefficient, as it takes up to ten generations of inbreeding following a cross to achieve a high level of homozygosity (Kasha and Maluszynski, 2003). Current single-seed-descent (SSD) methodologies enable a maximum of three generations per year to be developed in pea (Ochatt and Sangwan, 2010). Decreasing the length of the generation cycle will assist in overcoming this breeding bottleneck and result in the accelerated genetic improvement of pea.

Doubled haploidy has been utilised in important crops such as, tobacco (*Nicotiana tabacum* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and canola (*Brassica napus* L.) for the rapid achievement of homozygosity (Maluszynski et
In responsive species, doubled haploid (DH) technology has enabled the delivery of improved cultivars significantly faster due to the development of homozygosity in a single laboratory-based generation. Doubled haploidy is thus the fastest known route to homozygosity (Chase, 1952; Maluszynski et al., 2003). Legumes in general have remained recalcitrant to this technology (Croser et al., 2006; Lülsdorf et al., 2011). In recent years, significant progress has been made toward a DH protocol from isolated microspores of pea (Croser et al., 2006; Ochatt et al., 2009), however our understanding of the mechanisms by which isolated legume microspores shift their developmental pathways to divide and differentiate is limited (Croser et al., 2005; Sidhu and Davies, 2005; Croser et al., 2006; Ochatt et al., 2009; Lülsdorf et al., 2011). Doubled haploidy is not currently used in any breeding program and the development of a pea DH protocol would improve the efficiency of the time-consuming, laborious and often inefficient conventional breeding methods (Germanà, 2011). Further progress in the development of robust DH protocols, including the expansion to unresponsive species such as legumes can be expected with a more thorough understanding of the processes involved in haploid embryogenesis.

In the absence of an efficient DH system, an in vitro based SSD system has been proposed as a method to accelerate generation turnover across a range of species (Franklin et al., 2000; Ochatt et al., 2002; Asawaphan et al., 2005; Zhang, 2007; Ochatt and Sangwan, 2008; Ochatt and Sangwan, 2010). This technique involves the shortening of generation time by culturing immature seeds developed after forced in vitro flowering. There have been three in vitro flowering protocols proposed for pea (Fujioka et al., 1999; Franklin et al., 2000; Ochatt et al., 2002); however, each of these protocols was developed for a limited number of early-flowering cultivars. To enable
the widespread use of the *in vitro* flowering technology within pea improvement programs, it is necessary to gain a better understanding of the effect of physiological mechanisms operating *in vitro* on the efficient and reproducible induction of flowering and seed-set across a range of pea genotypes.

It is clear the development of an efficient DH system in pea will provide a valuable breeding technique. Given the well-documented difficulties associated with the development of DHs in the *Fabaceae*, it appears prudent to concurrently investigate the development of a simple, reliable and widely applicable *in vitro* based SSD system. An *in vitro* SSD system will offer an alternative method for the rapid achievement of homozygosity by shortening each generation cycle. In light of the potential benefits of these techniques to pea breeding, the aim of the research within this thesis was twofold. Firstly, to develop an anther culture based protocol for the production of haploid plants and ultimately doubled haploid populations and secondly, to develop an *in vitro* based SSD system across a range of genetically diverse pea genotypes. The overall goal was to provide pea breeders with biotechnology tools to rapidly achieve a high level of homozygosity and, in the process, gain a better understanding of the physiological mechanisms involved in these processes.
1.2 Literature review

1.2.1 Pea background

1.2.1.1 Classification

Cultivated pea (*Pisum sativum* L.) is an annual plant in the genus *Pisum*, within tribe *Fabae* (syn. *Vicieae*) in the *Fabaceae* family (syn. *Leguminoseae*) (Fig. 1.1). Pea is grouped with faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medik.), grasspea (*Lathyrus sativus* L.) and chickpea (*Cicer arietinum* L.) as a cool-season food legume (Muehlbauer, 1996). Peas are diploid (2n=2x=14) and highly self-pollinating (Gritton, 1980). Peas can be conventional-leaved, or semi-leafless, in which the leaflets on the conventional type are replaced by tendrils (Khan and Croser, 2004) (Fig. 1.2).

**Figure 1.1** Evolutionary and taxonomic relationships within major clades of crop legume subfamily *Papilionoideae*. MYA: million years ago (adapted from Choi et al., 2004).

**Figure 1.2** Pea plants with conventional leaf type (a) and semi-leafless type (b).
1.2.1.2 Origin of cultivated pea

The earliest date for the discovery of domesticated pea in archaeological sites is 7,000 to 6,000 BC from Turkey to Iran, and 1,000 – 2,000 years later in Greece and Europe (Zohary and Hopf, 1973; Ambrose, 1995). The Near East, Central Asia including Afghanistan, the Mediterranean, and Ethiopia abound with primitive forms and are sources of genetic diversity (Khan and Croser, 2004). Although the wild progenitor of pea is uncertain, Zohary and Hopf (1973) proposed *P. sativum* subsp. ‘*humile*’ found in steppelike habitats, under open conditions not very different from those prevailing in the cultivated field. Changes with domestication included the development of larger seed, loss of pod dehiscence and a shorter compact habit (Davies, 1976).

1.2.1.3 Distribution and production

Peas are an important crop in many arable regions of the world. Cultivation of pea includes diverse types and systems widely dispersed around the world in temperate to elevated subtropical environments (Redden et al., 2005). It requires moderate temperatures in the range of 12-18°C with a relatively humid climate for optimum growth (Khan and Croser, 2004). Peas are a valuable break crop providing disease and weed control in cereal-based cropping systems. In addition, as with all legumes, a symbiotic relationship with *Rhizobium* bacteria in the soil enables pea to fix atmospheric nitrogen (ca. 181-262 Kg N/ha) (McCallum et al., 2000) and make it available to the succeeding crop. Thus, peas are a useful component in cropping rotations, providing overall economic value in the cropping system (Muehlbauer, 1996).

Peas can be classified into two main groups: those harvested at the green or immature stage of development (vegetable type peas) and those harvested at the
physiologically mature stage (field or dry peas) (Redden et al., 2005). Green peas are one of the most popular vegetable items throughout the world, while dry peas are mostly used in the feed industry, particularly in the diet of pigs and poultry (Khan and Croser, 2004). Dry peas are also used as a whole in confectionery and snacks, milled to produce split peas for making soups, flour, and canned products (Khan and Croser, 2004). In Eastern Europe and Canada, peas are also grown for fodder or for green manure (Makasheva, 1983). Pea seeds are a good source of dietary protein and energy, containing 18-30% protein, 30-50% starch and 4-7% fibre. Pea protein is deficient in sulphur-containing amino acids, but contains relatively high levels of lysine making it a good dietary complement to cereals (McPhee, 2003).

In 2010, the world production of dry peas was 10.13 million tonnes (FAOSTAT, 2012). Major producers were Canada (28.2%), Russia (11.8%), France (10.8%) and China (8.8%). Australia is the eighth largest dry pea producer globally with 2.7% of the total production (FAOSTAT, 2012) (Fig. 1.3). Pea is the third most important pulse crop in Australia after lupin (Lupinus angustifolius L.) and chickpea, where in 2010, the pea cultivated area was 277,000 ha with a production of 280,000 Tonne and an average yield of 1.01 T/ha (FAOSTAT, 2012). The majority of the Australian product is exported, mainly for human consumption in Asia and the Middle East and to stockfeed markets in Asia and Europe (Pulse-Australia, 2012).
Figure 1.3  Dry pea production by country in 2010 (percentage of total global production). Source: FAOSTAT, 2012.

1.2.2 Genetic improvement in pea

1.2.2.1 Pea yield and breeding constraints

A major remaining challenge for pea breeders is the increase of yield potential and stability. Dry peas have high yields potentially reaching 8 tonnes per hectare (Cousin, 1997). However, the world average dry pea yield has remained relatively unchanged over the past 25 years, varying seasonally between 1.5 and 2.0 tonnes per hectare, on average (Fig. 1.4) (FAOSTAT, 2012). Many biotic and abiotic factors limit pea production. Diseases are considered the most important causes of reduced biomass production and seed yield. Abiotic stresses caused by adverse environmental conditions such as heat, drought and frost are also responsible for heavy pea production losses (Ali et al., 1994).
The development of improved germplasm in pea has been slow due to the low level of resistance/tolerance found in the available germplasm collections, low reliability of screening methods and polygenic nature of inheritance (Wroth, 1998). Over the years, biotechnology has emerged as a promising tool to overcome stresses in plants, but to date, progress has been limited in pea and legumes in general (McPhee, 2003; Dita et al., 2006). Peas have been described as recalcitrant in tissue culture and to biotechnological approaches (Ochatt et al., 2000), including doubled haploidy (Croser et al., 2006; Dita et al., 2006). Tissue culture protocols developed to date are either genotype-specific, hence not widely applicable, or they are slow and inefficient (Ochatt et al., 2000; Sidhu and Davies., 2005; Croser et al., 2006). In addition, in vitro root development can be difficult, often requiring several subculture steps (Tzitzikas et al., 2004) or grafting procedures (Bean et al., 1997). The development of biotechnology approaches in pea such as marker assisted breeding, tissue culture techniques, mutagenesis, and genetic transformation would assist breeders to overcome major biotic/abiotic constraints (Christou, 1993; Dita et al., 2006; Smýkal et al., 2012). The more efficient tissue culture techniques recently
established for many legume species should encourage legume researchers to resume
the use of techniques such as doubled haploidy, wide hybridisation and mutagenesis
in breeding programs (Dita et al., 2006).

1.2.2.2 Genetic improvement techniques

Various techniques are used in conventional pea breeding programs, and their
application depends on the type and amount of genetic variation in crosses, objectives
of breeding, and available genetic sources (Khan and Croser, 2004). Breeding
strategies in pea generally involve hybridisation among cultivars or between cultivars,
landraces or primitive forms, followed by combinations of pedigree selection, bulk
population, backcross, or single-seed-descent (SSD) methods of selection (McPhee,
2003; Redden et al., 2005).

In traditional breeding, the development of plant varieties with resistance to
abiotic and biotic stress is a very costly and time consuming process (Germanà, 2006).
In addition, a bias of dominance on the phenotype is created due to the selection of
individuals in the early stages of the process, where the non-competitive ones are
rapidly discarded. This early selection is based on individuals grown in non-crop
conditions without replication, as intensive selection cannot be applied until lines
approach homozygosity and sufficient seed is available for field trials (Maluszynski et
al., 2003; Thomas et al., 2003; Germanà, 2006). To overcome this problem, breeders
may delay intense selection until individual genetic lineages approach homozygosity
(Caligari et al., 1987).

Two in vitro technologies have been utilised in economically important crops for
the rapid achievement of homozygosity, and thus acceleration of the breeding
process:
1.2.2.3 Double haploidy

1.2.2.3.1 Introduction

The term ‘haploid plant’ refers to a plant derived from either the male (androgenesis) or female (gynogenesis) gametes. As there is no fertilisation between pollen and egg, the resulting haploid plants have only a single copy of the genetic information of the plant. When the chromosome complement is chemically or spontaneously doubled, these haploid plants become fertile doubled haploids (DHs) (Jain et al., 1996-1997; Maluszynski et al., 2003; Wędzony et al., 2009). The production of haploids and DHs through gametic embryogenesis allows the development of completely homozygous lines from heterozygous parents in a single laboratory-based step, shortening the time required to produced homozygous plants compared to conventional breeding methods that employ several to many generations of self-pollination. Doubled haploidy is thus the fastest route to homozygosity (Chase, 1952; Maluszynski et al., 2003).

In responsive species, DH technology has now become the most widely-applied and beneficial biotechnological tool for crop improvement (Dunwell, 2010). Its use in cereal and oilseed breeding programs has led to impressive gains in productivity (Jain et al., 1996-1997; Maluszynski et al., 2003). More than 280 varieties have been
produced with the use of various DH methods in crops including barley (*Hordeum vulgare* L.), asparagus (*Asparagus officinalis* L.), wheat (*Triticum aestivum* L.), canola (*Brassica napus* L.), rice (*Oryza sativa* L.) and tobacco (*Nicotiana spp.*) (Germanà, 2011).

Despite the development of protocols for more than 200 species, only four species (canola, tobacco, barley and wheat) are responsive enough to be used as model systems for the study of androgenesis elicitation (Touraev et al., 1997; Maraschin et al., 2005). Other scientifically or economically interesting species like *Arabidopsis thaliana* (L.) Heynh. and members of the *Fabaceae* family remain recalcitrant to doubled haploidy and little is known about the reasons for that (Croser et al., 2006; Dita et al., 2006; Seguí-Simarro and Nuez, 2008; Lülsdorf et al., 2011).

The poor fundamental understanding of the molecular and biochemical basis for plant gametophyte to sporophyte transition and morphogenesis makes the current empirical efforts to adapt DH production techniques to recalcitrant species highly time-consuming and difficult (Lülsdorf et al., 2011). The establishment in recent years of efficient *in vitro* androgenesis systems in species like canola and tobacco has opened the possibility of investigating the mechanisms underlying the switch from normal gametophytic pollen development towards the alternative sporophytic pathway and to isolate and characterise the genes and gene products involved in the regulation of this process (Touraev et al., 1996). Further improvement in existing DH protocols, and expansion to more recalcitrant species such as legumes, can be expected with the growing understanding of the processes involved in haploid embryogenesis.
1.2.3.2 Production of doubled haploids (DHs) via androgenesis

Several methods for haploid induction have been successfully used in many species. Modified pollination methods *in vivo*, such as wide hybridisation (interspecific and intergeneric hybridisation) and chromosome elimination (*H. bulbosum* technique), have been extensively used in cereal breeding programs (Dunwell, 2010). In addition, various methods have been developed for the *in vitro* development of haploid plants through the culture of immature gametophytes (gynogenesis and androgenesis) (Germanà, 2011). Androgenesis is the most deeply studied and the most widely effective technology (Wędzony et al., 2009). It is based on culture of male organs (anthers) or gametes (microspores). Under certain optimised conditions, microspores can shift development from gametophytic cells to sporophytic cells and then on to form haploid derived embryos. Inducing divisions and cell differentiation to produce such embryos is modulated by factors including genotype, growth conditions of donor plants, developmental stage of microspores, pre-treatment of flower buds and culture medium (Jain et al., 1996-1997; Dunwell, 2010; Germanà, 2011) (Fig. 1.5).

The culture of extracted anthers is the preferred method for DH production in many crops due to its simplicity, which permits large scale experiments in a wide range of genotypes (Kush and Virmani, 1996; Sopory and Munshi, 1996). The culture of isolated microspores requires better equipment and more skills compared to the culture of anthers. The isolation of pollen grains, asepsis, viability of the isolated pollen, culture vessels, and desiccation are all factors that need to be considered (Heberle-Bors, 1989). The advantage of the culture of isolated microspores is that it provides a better method for investigating cellular, physiological, biochemical and molecular processes of cellular totipotency using isolated single cells that can be readily staged for analysis (Nitsch, 1977; Reynolds, 1997).
Figure 1.5 Induction of microspore embryogenesis in *B.napus*. Culture of late unicellular microspores or early bicellular pollen at 32°C leads to embryogenic development. Culture at 18°C allows pollen maturation to proceed *in vitro* (modified from Cordewener et al., 1996).

Different species, as well as different cultivars within a species, have diverse requirements for androgenesis induction and no single standard protocol has been developed. Despite this, a number of general steps have been identified (Croser et al., 2006; Ferrie and Caswell, 2011; Germanà, 2011). Firstly, responsive genotypes and appropriate growing conditions for the production of healthy donor plants must be identified. These genotypes can then be used to optimise the conditions for the protocol, prior to widening its application to other genotypes. The pollen developmental stage is one of the most crucial factors of the culture procedure as small differences in developmental age can significantly affect the androgenesis induction response (Dunwell, 2010). The exact pollen developmental stage for best androgenesis induction varies with species. This period has been defined between the early uni-nucleate and early bi-nucleate stage of pollen development (Sangwan and Sangwan-Norreel, 1996).

Once microspores at the right developmental stage have been identified, a trigger needs to be developed to induce the switch from a gametic to a sporophytic pathway. Triggers are usually stress factors such as low or high temperature pre-
treatment of immature flower buds, carbon starvation pre-treatment, osmotic shock and centrifugation (Jain et al., 1996-1997; Touraev et al., 1997; Croser et al., 2006; Shariatpanahi et al., 2006; Wędzony et al., 2009). Other important factors that require optimisation include: media composition for microspore culture, embryo induction and maturation, plant regeneration, medium osmolarity, culture temperature, light intensity and culture container (Jain et al., 1996-1997; Wędzony et al., 2009; Germanà, 2011).

1.2.2.3.3 Role of abiotic stress treatments on androgenesis elicitation

Abiotic stress treatments play a key role in the induction of androgenesis as was first established for tobacco (Duncan and Heberle-Bors, 1976; Heberle-Bors and Reinert, 1981). Stress agents are widely used to stimulate the switch from gametic to sporophytic developmental for induction of microspore embryogenesis (Jain et al., 1996-1997; Touraev et al., 1997; Shariatpanahi et al., 2006; Wędzony et al., 2009). There is a large variety of proposed stress factors, with a review by Shariatpanahi et al. (2006) listing 16 different stresses. Stress can be applied via the donor plant, to immature flower buds prior to isolation and culture of anthers or microspores, or to anthers or microspores post-culture using nutritional, physical or chemical means. Physical stress treatments can be applied via temperature, atmospheric pressure, centrifugation and photoperiod, whereas chemical stress treatments can include carbon starvation of flower buds, anther or pollen, and ethanol treatments. Table 1.1 shows the most commonly applied stress treatments and their proposed mechanisms of action.
Table 1.1 Most commonly applied stress treatments for androgenesis induction and their proposed mechanisms of action.

<table>
<thead>
<tr>
<th>Stress treatment</th>
<th>Proposed mechanism of action</th>
</tr>
</thead>
</table>
| **Cold pre-treatment**| • Slows down degradation processes in the anther tissue thus protecting microspores from toxic compounds released in the decaying anthers (Duncan and Heberle-Bors, 1976).  
                          • Assures survival of a greater proportion of embryogenic pollen grains compared to heat treatment (Sunderland and Roberts, 1979).  
                          • Increases frequency of endo-reduplication leading to an increased number of spontaneous DH plants (Amssa et al., 1980).  
                          • Microspores in cold-treated anthers disconnect from the tapetum resulting in starvation (Sunderland and Xu, 1982).  
                          • Favours the synchronisation of the developmental process of microspores (Hu and Kasha, 1999).  
                          • Induces many morphological and physiological rearrangements in plant cells as well as hormonal changes (Żur et al., 2008). |
| **Heat shock**         | • Shown to cause changes in microtubule and cytoskeleton in cultured Brassica microspores (Hause et al., 1993; Cordewener et al., 1994; Simmonds and Keller, 1999).  
                          • Induces synthesis of heat-shock-proteins (HSP) in microspores (Pechan et al., 1991; Cordewener et al., 1995; Seguí-Simarro et al., 2003). |
| **Carbon starvation**  | • Cytoplasmatic and nuclear changes observed in starved microspores (Kyo and Harada, 1990; Garrido et al., 1995).  
                          • Decrease of RNA synthesis and protein kinase activities observed in tobacco microspores (Zarsky et al., 1990; Garrido et al., 1993). |
| **Colchicine**         | • Colchicine, a microtubule-depolymerizing agent, appears to be related to the disruption of the asymmetric spindle formation in gametophyte development and subsequent failure of cell wall formation which could permit fusion of the two nuclei and thus chromosome doubling (Kasha, 2005). |
| **Osmotic stress**     | • Accumulation of ABA observed in osmotic stressed tobacco (Imamura and Harada, 1980) and barley (Van Berger et al., 1999) anthers which may be involved in the reprogramming of microspore development (Wang et al., 2000; Żur et al., 2008).  
                          • Improves callusing from microspores, by favouring a slight detachment of membranes from the microspore wall facilitating initial division, and induces differentiation (Ochatt et al., 2009). |
| **Electroporation**    | • Shown to stimulate DNA synthesis in cultured plant protoplasts (Rech et al., 1988).  
                          • Creates temporary pores that allow nutrients from the culture medium to get into the cells (Zimmermann et al., 1976; Kinoshita and Tsong, 1977), thus improving regeneration competence from microspores (Delaitre et al., 2001; Ochatt et al., 2009) and protoplasts (Rech et al., 1987; Ochatt et al., 1988) of various species. |
| **Centrifugation**     | • Affects auxin to cytokinin ratios, thus facilitating the induction of embryos from microspores of tobacco and fourfold increasing the haploid plant regeneration rate (Tanaka, 1973). |
Upon stress treatment, isolated microspores swell and their cytoplasm undergoes structural reorganization. The nucleus moves to a more central position and cytoplasmic strands are formed that pass through the vacuole and connect the perinuclear cytoplasm with the subcortical cytoplasm (Touraev et al., 1997). Induction brings on an altered synthesis and accumulation of RNA and proteins in potentially embryogenic microspores, leading to the sporophytic divisions (Reynolds, 1997). The induction of sporophytic development is only possible at early developmental stages, when the gametic cell appears to be totipotent (Touraev et al., 2001).

The induction of androgenesis through the application of stress treatments has been linked with changes in endogenous hormone content (Wang et al., 2000; Seguí-Simarro and Nuez, 2008; Żur et al., 2008; Lülsdorf et al., 2012). Accumulation of abscisic acid (ABA) during androgenesis induction has been observed in a number of species after the application of stress treatments (Imamura and Harada, 1980; Van Berger et al., 1999; Żur et al., 2008). Lülsdorf et al. (2012) observed that androgenesis-inducing stress treatments in pea, chickpea and lentil anthers caused a greater proportional increase in active auxins, particularly indol-3-acetic acid-asparagine (IAA-asp), compared to the other hormones measured. They also observed that the concentration of ABA and its catabolites spiked in control and cold-treated anthers and decreased after osmotic shock treatment in the three species studied. Also, no bioactive gibberellins (G₃, G₅, G₄ and G₇) were detected in this study for any of the studied species, indicating that this hormone group is likely not linked to androgenetic elicitation in the legumes.

To date, the mechanism for the developmental switch of microspores from gametophytic to sporophytic development has not been established for any species
However, over the last few years, significant progress has been made toward the discovery of genes involved in haploid embryogenesis and their mechanisms of action, mostly from three model species: canola, tobacco and barley (Boutilier et al., 2005; Hosp et al., 2007). Also, the sequencing of the *Arabidopsis* genome has permitted the isolation of genes related to embryo induction in microspores and their subsequent tagging and isolation in crop species (Kasha, 2005).

Functional genomics approaches have revealed several hundreds of up-regulated and down-regulated genes associated with androgenic induction (Hosp et al., 2007). The induction of changes in development is characterised by the activation of specific transcription factors, which in turn cause an altered pattern of gene expression. In several cases these genes appear to be stress-related (Elmaghrabi et al., 2013) or associated with zygotic embryogenesis (Verdier and Thompson, 2008; Elmaghrabi et al., 2013). The stress-related genes may be concerned with a general reprogramming of the cell or providing some type of protection from that stress (Raghavan, 1986). This involves a profound remodelling of the transcriptome and up-regulation of genes involved in primary metabolism and biosynthesis of lipids, carbohydrates and proteins (Gallardo et al., 2007; Seguí-Simarro and Nuez, 2008; Verdier and Thompson, 2008; Elmaghrabi et al., 2013). Other gene groups related to mitochondria, cytoskeleton, epigenetic chromosomal remodelling, signal transduction and stress response are also up-regulated when compared with non-embryogenic commitment (Seguí-Simarro and Nuez, 2008). In both tobacco and canola, a close relationship has been observed between the induction of embryogenesis and the expression of heat shock proteins (HSP) (Cordewener et al., 1998; Pechan and Smýkal,
There are many small HSPs produced by stress, with HSP70 being the predominant one in treated microspores (Kasha, 2005).

Despite the significant progress made in the last few years toward the discovery of the genes involved in haploid embryogenesis and their mechanisms of action, the genetic basis of the process of doubled haploidy is still largely unknown. The new approaches in discovering gene function are expected to have an impact in the future (Wędzony et al., 2009). Further progress in the identification and characterisation of genes specifically expressed during early stages of microspore embryogenesis will lead to a better fundamental understanding of the mechanisms involved in androgenic induction and the subsequent formation of haploid plants. This will have a positive impact in the development of improved protocols in many plant species, particularly in legumes. For the moment, further improvements in microspore/ anther culture protocols can be expected mostly from empirical testing of new variants of known protocols and from the optimisation of factors identified as critical in androgenic development.

1.2.2.3.4 Application of haploids and DHs in plant breeding, genetics and functional genomics

The instant production of true-breeding lines in diploid or allopolyploid species saves a number of generations in the breeding program. With this technology it is possible to cross parental lines and conduct field trials on DH derived progeny within two years (Thomas et al., 2003) and in self-pollinating crops such as pea, cultivar developmental time can be reduced by up to five years. The homozygous nature of the DH population ensures a more efficient and reliable selection compared to conventional early-generation segregating lines as there are no dominance-related effects (Thomas et al., 2003). Also, quantitative trait evaluation can begin earlier in the
program, saving time and space (Kasha and Maluszynski, 2003). Using a combination of backcrossing, marker assisted selection and doubled haploidy, one can rapidly introduce new genes into a variety. Due to these advantages, DH technology is currently an integral part of the breeding programs of agronomically important crops such as wheat, barley and canola (Wędzony et al., 2009; Germanà, 2011).

In addition to the exploitation of DH lines in practical breeding, they have widespread application in such research areas as gene mapping and genomics, mutation studies, and as targets for transformation. The advent of molecular markers revolutionised the construction of genetic maps of plant genomes and doubled haploidy has played a major role in facilitating the generation of maps in a range of species, notably barley, rice, canola and wheat (Forster and Thomas, 2005). Doubled haploid populations are ideal for genetic mapping as a population of recombinant inbred lines (RILs) can be ready for DNA extraction and mapping less than two years after the initial cross. These RILs can be re-grown and distributed in seed form with certainty as to their homozygosity making it easier to screen with a large number of markers (Forster and Thomas, 2003; Lionneton et al., 2004). Map construction from a DH population derived from the F\textsubscript{1} of a cross is relatively simple because the expected segregation ratio is the same as that of a backcross (Snape, 1976).

Microspores provide an excellent single-celled target for genetic manipulation through mutation or transformation, enabling enhancement of genetic variability (Szarejko, 2003). The single-celled nature of the starting material means that chimerae or heterozygosity are avoided and the mutation/ transgene is expressed throughout the entire plant (Germanà, 2011). Since the advent of haploid systems, their use for mutant induction and selection has been listed among its most important applications.
This approach has been undertaken for the selection of lines with high photosynthetic activity in haploid tobacco plants (Medrano and Primo-Millo, 1985), for the isolation of disease resistance in melon (*Cucumis melo* L.) (Kusuya et al., 2003), and for isolation of dwarf potato (*Solanum tuberosum* L.) mutants with gibberellin biosynthesis deficiency from anther culture-derived potato lines (Valkonen et al., 1999). More recently studies have been conducted to select lines tolerant to oxidative stress in DH maize (*Zea mays* L.) derived from microspores exposed to paraquat (Darko et al., 2009) and to isolate lines with increased aluminium tolerance in aluminium-treated wheat anther cultures (Bakos et al., 2008).

Transformation and DH technology have been used to create homozygous lines for the trait of interest, thereby speeding up the breeding process (Ferrie and Möllers, 2011). Isolated microspores not only provide a good target for bombardment but also are readily amenable to transgene *in vitro* selection (Shim and Kasha, 2003). In 1994, Jähne et al. were the first to obtain homozygous plants for the transgenes using biolistic bombardment of freshly-isolated barley microspores. Transgenic embryos or calli can be selected from cultures by using resistance selection, either to herbicides or to an antibiotic resistance marker or with a visual marker gene such as the green fluorescent protein (GFP) (Shim and Kasha, 2003). Transformation and DH technology have permitted the creation of homozygous *Brassica* lines with enhanced tolerance to clubroot (*Plasmodiophora brassicae*) (Reiss et al., 2009) or pollen beetle (Åhman et al., 2006).

### 1.2.2.3.5 Pea doubled haploid research status

The production of DHs via androgenesis should be feasible in most plant species but it has taken a long time to develop protocols robust enough to be used in
breeding programs and these have been developed for a limited number of crops. Each crop has different requirements and thus, the need for extensive research to develop an efficient DH system (Kasha and Maluszynski, 2003). Leguminous species, including pea, have been described as recalcitrant to DH techniques (Croser et al., 2006) and progress towards this goal has been very slow (Table 1.2). In 1972, Gupta et al. were the first to report haploid callus induction from anthers of pea breeding line B22. Microspores were at the uni-nucleate stage at the moment of culture. No plant regeneration was achieved nor was the ploidy level of the callus cells confirmed. A few years later, Gupta (1975) reported the production of a few roots, shoots and torpedo-shaped embryos, again with no confirmation of ploidy level. Gosal and Bajaj (1988) successfully induced callus by applying a cold pre-treatment to extracted anthers of the pea cv. Bonneville and the breeding lines T163 and P88. A few heart-shaped embryos were developed but no plant regeneration was obtained. About 90% of the cells were diploid indicating that callus might have developed from maternal anther tissue rather than from microspores.

More recently, Sidhu and Davies (2005) cultured anthers of cvs. Mukta, Gorokh 40, Pelican and P. fulvum genotype ATC113 using media supplemented with casein hydrolysate and various plant growth regulators. Thirty to 40% of anthers produced callus and two putative haploid/DH shoots were recovered, one each from cvs. Gorokh and Pelican. In pea cultivars CDC April and Highlight a cold pre-treatment of buds coupled with electroporation of isolated microspores led to symmetrical microspore nuclear divisions and early-stage embryo production (Croser and Lülsdorf, 2004; Croser et al., 2005; Croser et al., 2007; Grewal et al., 2007). A combination of multiple stress factors including cold-pre-treatment of buds, osmotic and electric
shock, successfully improved androgenetic divisions from extracted anthers (Ochatt et al., 2007) and isolated microspores (Ochatt et al., 2009) of cultivars Frisson, Terese, Victor, Cheyenne and CDC April. Calli and haploid derived embryos were obtained and a few weak plantlets recovered. The haploid condition of these materials was confirmed by flow cytometry. In 2010, Bobkov reported the development of callus tissues and globular embryoids in pea anther culture on a medium containing 2,4-D after a cold (4°C) treatment of buds but no haploid plants were recovered with this protocol.

Despite the recent encouraging results, no DH protocol has been developed for pea or any other cool-season legume crop that can be routinely exploited in a breeding program. The fundamental understanding of the molecular and biochemical basis for microspore developmental transition from gametophytic to sporophytic pathway remains elusive (Croser et al., 2006; Ochatt et al., 2009; Germanà, 2011; Lülsdorf et al., 2011). More research is therefore required to improve our understanding of the molecular and biochemical mechanisms involved in androgenesis elicitation in order to facilitate the development of a robust protocol that can be applied in extensive pea breeding programs and genetic studies.
Table 1.2 Summary of key publications on haploid research in pea (AC: anther culture; IMC: isolated microspore culture).

<table>
<thead>
<tr>
<th>Author/s</th>
<th>Target</th>
<th>Cultivar/line</th>
<th>Stress treatment</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Gupta et al. 1972</td>
<td>AC</td>
<td>B22</td>
<td>--</td>
<td>Callus formation</td>
</tr>
<tr>
<td>2 Gupta 1975</td>
<td>AC</td>
<td>B22</td>
<td>--</td>
<td>Few shoots/ torpedo-shaped embryos; No plants recovered</td>
</tr>
<tr>
<td>1 Gosal and Bajaj 1988</td>
<td>AC</td>
<td>Boneville, T163 and P88</td>
<td>Anthers: Cold for 72 h</td>
<td>Callus/ heart shaped-embryos</td>
</tr>
<tr>
<td>2 Croser and Lülsdorf 2004</td>
<td>IMC</td>
<td>CDC April; Highlight</td>
<td>Buds: Cold or heat</td>
<td>Symmetrical microspore nuclear divisions to multinucleate stage</td>
</tr>
<tr>
<td>3 Sidhu and Davies 2005</td>
<td>AC</td>
<td>Gorock 40; Pelican</td>
<td>Anthers: Cold for 72 h</td>
<td>Callus production; Two putative haploid shoots recovered</td>
</tr>
<tr>
<td>3 Croser et al. 2005; 2 Croser et al. 2007</td>
<td>IMC</td>
<td>CDC April; Highlight</td>
<td>Buds: Cold for 48 h</td>
<td>Symmetrical microspore nuclear divisions; Early-stage embryos</td>
</tr>
<tr>
<td>2 Grewal et al. 2007</td>
<td>IMC</td>
<td>CDC April</td>
<td>Buds: Cold for 4 d Microspores: electroporation</td>
<td>Multinucleate microspores; Pro-embryos</td>
</tr>
<tr>
<td>2 Ochatt et al. 2007</td>
<td>AC</td>
<td>Frisson; Terese, Victor; Cheyenne</td>
<td>Buds: Cold &gt; 48 h Anthers: osmolarity and electroporation</td>
<td>Cali, somatic embryos and buds; A few enfeebled plantlets recovered</td>
</tr>
<tr>
<td>1 Ochatt et al. 2009</td>
<td>IMC</td>
<td>Frisson; Terese; Cameor; Frisson x Cameor</td>
<td>Buds: cold for 2-30 d Microspores: osmolarity and electroporation</td>
<td>Few flow cytometry confirmed doubled haploid plants</td>
</tr>
<tr>
<td>1 Bobkov 2010</td>
<td>AC</td>
<td>Faraon, Vizir, Mul'tik, Madonna, Stabil and BL101</td>
<td>Buds: Cold Anthers: Heat</td>
<td>Green morphogenetic calli; Globular embryoids</td>
</tr>
</tbody>
</table>

1 Journal publications  
2 Non refereed conference proceedings  
3 Refereed conference proceedings
1.2.2.3.6 Application of flow cytometry in plant breeding

Flow cytometry is a high-throughput analytical tool that simultaneously detects and quantifies multiple optical properties (fluorescence, light scatter) of single particles, usually cells or nuclei labelled with fluorescent probes, as they move in a narrow liquid stream through a powerful beam of light (Kron et al., 2007). Flow cytometry facilitates a wide range of parameters to be simultaneously recorded from individual particles, in contrast to other techniques that generate average values of the whole population of measured particles (Doležel et al., 2007a). Also, relationships between different properties can be determined after examining multiple parameters for each cell thus providing a better understanding of the factors responsible for the characteristics of cells and cell cultures (Yanpaisan et al., 1999). As a result, flow cytometry is now used routinely in a number of fields ranging from basic cell biology to genetics, immunology, molecular biology, and environmental science (Kron et al., 2007).

Flow cytometry has been used for the analysis of the composition of various chemical components of different tissues (Cvikrová et al., 2003; Loureiro et al., 2006), or cell wall fractions (Ochatt, 2006), chromosome sorting for physical mapping of genomes (Ng and Carter, 2006; Doležel et al., 2007b; Ng et al., 2007), characterisation of true-to-typeness (Geoffriau et al., 1997; Roux et al., 2003; Elmaghrabi and Ochatt, 2006; Kone et al., 2007) and for the distinction between closely related genotypes (Ochatt et al., 2013). This tool has also been used as a predictor of regeneration competence from single-cell derived cultures (Ochatt et al., 2000; Ochatt et al., 2010) or of the occurrence of hyperhydricity in regenerants (Ochatt et al., 2002), as well as
for the assessment of developmental status of immature seeds and embryos (Atif et al., 2013).

In terms of determination of ploidy level in plants, chromosome counting using root tip cells arrested in metaphase remains the unambiguous method to define the chromosomal complement. However, this technique is very time-consuming; it requires highly skilled operators and the supply of tissues containing discrete numbers of dividing cells, which may not be easily available (Doležel et al., 2007a; Ochatt, 2008; Ochatt et al., 2011). Alternative methods that do not require mitotically-active cells include leaf stomatal density and size (van Duren et al., 1996) or the size of pollen grain (Zonneveld and van Iren, 2001) and cell size (Hao et al., 2002). However, these techniques have generally proved to be insufficiently reliable (Ochatt, 2008). Since the nuclear DNA content correlates with ploidy, a high-throughput solution was provided by the flow cytometry estimation of DNA content (Bohanec, 2003; Doležel et al., 2007a; Ochatt, 2008). Flow cytometry has been extensively used to efficiently determine the relative nuclear DNA content and ploidy level across a large number of species including the legumes pea, *Medicago truncatula* Gaernt. and *Lathyrus* spp. (Ochatt et al., 2009) and chickpea (Grewal et al., 2009). When optimised protocols are used, a high number of samples, 100 or more per day, can be accurately analysed (Bohanec, 2003; Cousin et al., 2009).

Despite the wide applications of flow cytometry to plant cell analysis, its use for the analysis of embryogenic potential in plant cell populations has been relatively limited (Ochatt, 2008). Flow cytometry has been used to study the embryogenic potential from protoplasts of pea (Ochatt et al., 2000) and cultured microspores of canola (Schulze and Pauls, 1999; Schulze and Pauls, 2002) and brown mustard...
Brassica juncea L. Czern.) (Lionneton et al., 2001). Flow cytometry promises quantitative and qualitative advances in the understanding of plant growth, development and function at subcellular, cellular and organismal levels (Doležel et al., 2007a), and particularly of the factors affecting haploid embryogenesis.

1.2.2.4 In vitro based Single-Seed-Descent (SSD)

1.2.2.4.1 Introduction

Given the obvious difficulty inherent in the development of DH technology in pea, I decided to concurrently explore an alternative in vitro approach for the rapid achievement of homozygosity. The in vitro based modified SSD system has been proposed as an alternative method to accelerate generation turnover across a number of species. Conventional SSD consists of randomly selecting one seed per plant from an F₂ population following a cross, and advancing it through the early segregating generations, thus permitting the rapid fixation of genes in breeding lines (Goulden, 1939). As only one seed is needed to produce the next generation, plants can be grown under conditions that accelerate flowering and seed-set, but do not encourage high yield. The technique of SSD makes it possible to advance a population to a homozygous generation in a much shorter time than the conventional plant breeding methods, which generally use one generation per year (Brim, 1966). Current conventional SSD methodologies enable a maximum of three generations per year to be developed in pea breeding programs (Ochatt and Sangwan, 2010), provided breeders have access to controlled temperature environments. The in vitro SSD technique involves the shortening of generation time by culturing immature seeds developed after forced in vitro flowering under conditions for accelerated flowering.
and seed-set. This leads to rapid generation turnover by reducing the length of the generation cycle, enabling multiple generations per year.

1.2.2.4.1 \textit{In vitro} flowering in pea

\textit{In vitro} flowering has been reported in many plant species, including \textit{Amaranthus spp.} (Tisserat and Galletta, 1988), potato (Al Wareh et al., 1989), Japanese pear (\textit{Pyrus serotina} Rehd) (Tsujikawa et al., 1990), \textit{Kalanchoe blossfeldiana} Poelln. (Dickens and Van Staden, 1990), cranberry (\textit{Vaccinium macrocarpon} Ait. ‘Stevens’) (Serres and McCown, 1994), bamboo (\textit{Bambusa arundinacea} (Retz.) Willd.) (Joshi and Nadgauda, 1997), ginseng (\textit{Panax ginseng} C.A. Meyer) (Lin et al., 2005), \textit{Kniphofia leucocephala} Baijnath. (Taylor et al., 2005), \textit{Perilla frutescens} (L.) Britton (Zhang, 2007), \textit{A. thaliana} (Ochatt and Sangwan, 2008; Ochatt and Sangwan, 2010), and tomato (\textit{Lycopersicon esculentum} Mill.) (Bhattarai et al., 2009). However, a limited number of publications are available in legumes species e.g. soybean (\textit{Glycine max} (L.) Merr.) (Dickens and van Staden, 1985), peanut (\textit{Arachis hypogaea} L.) (Narasimhulu and Reddy, 1984; Chengalrayan et al., 1995; Asawaphan et al., 2005), \textit{Vigna mungo} L. (Ignacimuthu et al., 1997) and \textit{Vigna aconitifolia} (Jacq) Marechal (Saxena et al., 2008).

In pea, there have been three \textit{in vitro} flowering protocols proposed. In 2000, Franklin \textit{et al.} successfully obtained \textit{in vitro} flowering and seed set with cv. ‘PID’. In this protocol an \textit{in vitro} rooting phase, a reduced concentration of ammonium nitrate and the addition of auxins to the culture medium were key factors for the induction of flowering \textit{in vitro}. Ochatt \textit{et al.} (2002) designed a strategy to reduce the length of the generation cycles \textit{in vitro}. Neither adding plant growth regulators (1 mg/L of 1-naphtaleneacetic acid, NAA), nor reducing salt strength in the medium by half or the rooting of \textit{in vitro} shoots were essential to induce \textit{in vitro} flowering and seed-set.
Using this protocol, 6.87 generations per year were obtained with cv. ‘Frisson’ and 5.24 generations per year with cv. ‘Terese’. Fujioka et al. (1999) also studied the effects of culture conditions on in vitro flowering and seed-set for the Japanese pea cultivars ‘Misasa’ and ‘Kishu-usui’. They observed that by growing plants in 30 x 200 mm glass tubes covered with a cotton stopper and culturing at 25°C with a light intensity of 135 µmol m$^{-2}$ s$^{-1}$ and a photoperiod of 24 h, in vitro flowering could be achieved in 34.6 days for cultivar ‘Misasa’ (100% pod-set) and in 70.8 days for cultivar ‘Kishu-usui’ (57.7% pod-set). Whilst providing an excellent platform for future studies, these protocols were developed only for early-flowering pea cultivars. The identification of a simple, robust and widely applicable in vitro system to accelerate generation turnover by shortening the length of each cycle will be of great value in pea breeding programs.

1.2.2.4.3 Factors affecting flowering in pea

The pea plant is botanically indeterminate and continues to grow provided sufficient moisture is available (McPhee, 2003). The first nodes, some of which give rise to branches, are vegetative, while the subsequent nodes are reproductive. Generally two flowers, from which the pods develop, are present at each reproductive node (Cousin, 1997). The flowering habit of peas is a genetic characteristic of each variety. All known genotypes produce a number of vegetative nodes before flowering, ranging from as few as four in the earliest varieties to over 100 in the latest varieties under non-inductive conditions (Weller et al., 1997). The onset of flowering and the number of flowering nodes is triggered by environmental conditions, such as temperature, light quality, photoperiod, carbon dioxide level, humidity and nutrients (Gottschalk, 1988; Cerdan and Chory, 2003; Iannucci et al., 2008; Nelson et al., 2010).
Natural environment, and even glasshouse conditions can be highly variable making it difficult to examine the influence of environmental factors on flowering (Cummings et al., 2007). Controlled environment growth chambers are commonly used in order to minimise environmental variation and to provide plants with the best conditions to accelerate flowering and seed-set.

Light and temperature are the main environmental factors that influence flowering in pea (Murfet and Reid, 1974; Moe and Heins, 1990; Nelson et al., 2010). Light provides environmental information for the plant and consequently affects a wide range of photomorphogenic responses, including germination, de-etiolation, elongation, leaf expansion and flowering (Weller, 2004; Spalding and Folta, 2005). Plants detect light quality by at least three families of photoreceptors: phytochromes, cryptochromes, and one or more unidentified ultraviolet light receptor(s) (Runkle and Heins, 2001). Phytochrome absorbance peaks are in the red light (R, 600-700 nm), and far-red (FR, 700-800 nm) and to a lesser extent in blue (B, 400-500 nm). Blue light is also absorbed by cryptochromes. As plants preferentially absorb R light for photosynthesis, the light under canopies or in crowded conditions has low red to far-red (R:FR) ratio, which can result in the shade avoidance response: elongation of stems, reduced leaf size and earlier flowering (Smith, 1994). Conversely, light rich in R light (high R:FR ratio) signals non-competitive conditions, and can result in relatively reduced plant height and later flowering (Runkle and Heins, 2001). A R:FR ratio of 1:1 seems to be the most effective for flower induction in long-day pea plants (Cummings et al., 2007).

The influence of temperature on flowering is mediated through at least two mechanisms in addition to the obvious effect on growth rate (Reid and Murfet, 1975).
Firstly, it is proposed that the inhibitor pathway has a higher temperature coefficient than the floral stimulus pathway leading to promotion of flowering by low temperatures. Secondly, low temperature (vernalisation) treatment seems to have a specific effect at the shoot apex rendering it more responsive to the flowering signal (Reid et al., 1996).

The growth photoperiod also has an important effect on the initiation of flowering in pea. Most pea cultivars are facultative long-day plants. However, some are day-neutral and others are essentially obligate long-day plants that may not flower at all in photoperiods shorter than 12 h if unvernalis ed (Murfet, 1985). In addition to the effect on flower initiation, photoperiod also affects a number of other characteristics in pea (Weller et al., 1997). Relative to long-day conditions, short-day conditions promote branching of the shoot and elongation of peduncles, and inhibit stem elongations, apical arrest and the growth of flowers and pods (Murfet, 1985; Murfet and Reid, 1993). Environmental gas composition (ethylene levels and carbon dioxide concentration) and humidity levels, through the use of different type of culture vessels and closure systems, have an important effect on flowering under *in vitro* conditions (Fujioka et al., 1999; Asawaphan et al., 2005). In 1999, Fujioka et al. observed a higher proportion of *in vitro* flowering in pea when a 30 x 200 mm test tube and a cotton stopper were used. In peanut, Asawaphan et al. (2005) observed a higher number of flowers produced *in vitro*, as well as an increase in carbon dioxide concentrations when culturing seedling in vessels with reduced ventilation. These results suggest that carbon dioxide levels may have a promoting effect on flowering. In contrast, in *Brassica campestris* L., *in vitro* accumulation of ethylene was observed when tightly sealed culture containers were used (Lentini et al., 1988). The
accumulation of ethylene in sealed containers was associated with inhibition of plant development, with the production of abortive flowers or no floral buds.

Along with environmental factors and physiological state of the plant, the use of different types of growth regulators has been shown to influence the induction of flowering in vitro in several species. The requirement of auxins for flower induction has been reported in many plants species, either alone (Ignacimuthu et al., 1997; Zhang, 2007) or in combination with cytokinins (Narasimhulu and Reddy, 1984). However, in some species auxins are either ineffective (Rastogi and Sawhney, 1987) or inhibitory (Deaton et al., 1984; Lin et al., 2005) for floral development. Cytokinins have also shown a promoting effect on in vitro flowering (Lin et al., 2005; Taylor et al., 2005). Gibberellins can promote seed germination, internode elongation and flowering (particularly in species that require long days and/or cold) (Gaspar et al., 1996). Gibberellins are essential for floral bud growth in general, and stamen development in particular (Sawhney and Rastogi, 1990). Gibberellic acid (GA₃) combined with auxins has been used to induce in vitro flowering in various species (Pharis et al., 1987; Evans et al., 1990; Sankhla et al., 1994), including pea (Franklin et al., 2000). However, Murfet and Reid (1987) affirmed there is no evidence to suggest gibberellins have any facilitating role in the flowering process as pea mutants with a severe block in the gibberellin synthesis pathway still flower.

1.2.2.4.3 Genetics of flowering in pea

Pea has been a model for the genetics of flowering for several decades and numerous flowering loci have been identified, but until recently little was known about the molecular nature of these loci. To date, more than 20 flowering-related loci have been identified in pea from natural genetic variation and induced mutations
(Weller et al., 2009). The ability to respond to photoperiod in pea is conferred by the action of three complimentary dominant genes Sn, Dne and Ppd (Barber, 1959; Murfet, 1971a; King and Murfet, 1985; Reid et al., 1996). The Sn Dne Ppd system is responsible for the formation of a graft-transmissible inhibitor of flowering under non-inductive, short day conditions, resulting in the delay of flower initiation (Murfet, 1971b; Murfet and Reid, 1973; King and Murfet, 1985; Reid et al., 1996; Taylor and Murfet, 1996). Two major genes, E and Hr, influence ontogenetic expression of the system. The dominant allele E reduces inhibitor synthesis in the cotyledons (Murfet, 1971b; Murfet, 1971c; Murfet and Reid, 1973; Reid et al., 1996; Taylor and Murfet, 1996), while Hr prolongs inhibitor synthesis in the shoot (Murfet, 1973; Reid, 1979; Reid et al., 1996). Flowering behaviour in pea is also determined by the genotype at the Lf locus, which acts at the shoot apex to determine sensitivity to the flowering signal (Murfet, 1971b). In addition to genes controlling inhibitor synthesis, a gene (Gi) has been identified in pea, which acts on the synthesis pathway of the floral stimulus (Beveridge and Murfet, 1996).

1.2.2.5 Doubled haploidy vs. Single-Seed-Descent

Plant breeders have always been interested in shortening the period for inbreeding. Both DH and SSD breeding methods attempt to reduce the time required for inbreeding by rapidly advancing generations without breeder or natural selections. Conventional breeding methods consist of several backcrossing or self-pollination steps, thus they are labour-intensive and time-consuming procedures. The SSD strategy was devised to speed-up the development of homozygous lines, but it suffers from the same handicaps as the conventional breeding technique of pedigree inbreeding in terms of time delays and competitive interactions among plants.
(Germanà, 2006). In contrast, gametic embryogenesis makes the production of homozygous lines feasible and shortens the time required to produce such lines, allowing the single-step development of completely homozygous lines from heterozygous parents (Germanà, 2011).

Production of DHs from an F1 hybrid limits the opportunity for recombination between loci to a single meiosis. In contrast, random inbred lines produced from the F2 generation by SSD will have passed through several rounds of potential recombination (Caligari et al., 1986). Hence, the linkage bias in F∞ samples produced by SSD will be less than that of similar samples produced by DH from the F1 (Caligari et al., 1987). In barley anther culture, a clear segregation distortion in the absence of selection was observed (Zivy et al., 1992). On the other hand, Thomas et al. (2003) affirmed that a comparison of theoretical properties of DH and SSD progenies from a range of species, principally cereals, showed that in the absence of linkage there is no difference between these two strategies (Thomas et al., 2003), so the choice of the strategy to adopt will depend on whether linkage blocks are to be preserved (F1DH) or broken-up (SSD). The adoption of doubled haploidy does not lead to any bias of genotypes in populations, and random DHs were even found to be compatible to selected lines produced by conventional methods (Winzeler et al., 1987; Jain et al., 1996-1997; Germanà, 2006).

Some researchers argue that the theoretical benefits are not enough to deploy DH technology in all breeding programs. Indeed, cost efficiency, fixation of rare and useful alleles, conservation of genetic variability in selection lines, uniformity and stability of new released varieties must be considered (Thomas et al., 2003; Germanà, 2006). The main difficulty in improving androgenesis lies in the large range of factors
that control and influence the process (Wędzony et al., 2009). Conversely, the SSD technique is very efficient on account of selection effort, is less expensive and labour intensive, and can be utilised across a wider range of genotypes. The SSD strategy offers the greatest benefits in situations where simultaneous selection is required for several characteristics with different heritability.

1.2.2.6 Benefits of pea research to the Australian industry sector

The pea production area has significantly decreased in the last few decades worldwide (FAOSTAT, 2012) predominantly as a result of the unstable yields caused by many biotic and abiotic limitations. Further adoption of pea by farmers is dependent on the availability of superior cultivars with improved abiotic and biotic stress resistance, which will lead to more reliable yield and quality. One of the main limitations to pea genetic improvement is the length of the cultivar development process and the absence of biotechnology tools applicable to the crop. This prevents a timely response to emerging threats and end-user’ needs. The development of in vitro technologies in pea, like doubled haploidy and in vitro SSD, has the potential to contribute exciting new dimensions to the conventional exploitation of genetic resources and breeding strategies for improved varieties (Redden et al., 2005). The availability of new biotechnology tools will help overcome some of the major constraints to increasing productivity in pea. By accelerating the development of better adapted cultivars these techniques will greatly assist pea breeders in the development of new germplasm and confer the ability to quickly respond to novel abiotic and biotic threats. Economically, this could lead to substantial increases in pea production area which would offset fertiliser and pesticide input costs and assist with maintaining viable margins in cropping-based agricultural operations. This would have
important social ramifications, enabling smaller farm operations to remain viable leading to critical population mass in rural areas to support important community infrastructure.

1.3 Research objectives and thesis structure

The aim of this research is to accelerate the breeding process in pea by developing *in vitro* methods to more rapidly achieve a high level of homozygosity and to gain a better understanding of the physiological mechanisms involved in these processes. Chapter 2 presents the work undertaken on the development of a DH protocol from extracted anthers in selected pea genotypes of varying genetic backgrounds. Chapter 2 includes the study of factors known to be involved in the elicitation of androgenesis. This Chapter leads into Chapter 3, which describes the flow cytometry study of the effect of abiotic stress factors on androgenesis elicitation from extracted anthers and isolated microspores of pea. In Chapter 4, an alternative method of obtaining rapid homozygosity is studied. This chapter focuses on the study of the effect of physiological mechanisms operating *in vitro* on the efficient and reproducible induction of flowering and seed-set across a range of pea genotypes for use in SSD breeding strategies. Finally, Chapter 5 provides a summary of the research undertaken and outlines proposed future directions.
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Chapter 2

The application of multiple stress treatments and the optimisation of culture conditions lead to routine induction of androgenesis from intact anthers of pea (*Pisum sativum* L.)

2.1 Introduction

Doubled haploid (DH) technology is a well-established technique for accelerating the genetic progress of key cereal, oilseed and horticultural crop species (Jain et al., 1996-1997; Wędzony et al., 2009; Dunwell, 2010; Germanà, 2011). The production of DHs through gametic embryogenesis allows the single-step development of completely homozygous lines from heterozygous parents, shortening the time required to produce homozygous plants in comparison to the conventional breeding methods that require generations of selfing (Germanà, 2011). Doubled haploidy is thus the fastest known route to homozygosity (Chase, 1952; Maluszynski et al., 2003). However, to date, no DH protocol is routinely used in any crop, pasture or tree legume breeding program, and a fundamental understanding of the mechanisms involved in the developmental transition of microspores from the gametophytic to the sporophytic pathway remains elusive (Croser et al., 2006; Ochatt et al., 2009; Germanà, 2011; Lülsdorf et al., 2011). It is therefore the aim of this research to study the effects of key factors known to be involved in androgenesis elicitation in order to develop a robust anther culture protocol across a range of pea (*Pisum sativum* L.) genotypes.

Haploid embryogenesis requires the reprogramming of microspores, diverting them from their original pathway, toward embryogenesis (Jain et al., 1996-1997). It is clear from the literature that this switch in development is achieved by the application
of stress treatments and the manipulation of in vitro culture conditions (Touraev et al., 1997; Shariatpanahi et al., 2006; Wędzony et al., 2009; Dunwell, 2010; Ferrie and Caswell, 2011; Germanà, 2011). Genotype, microspore developmental stage, and culture conditions are the most important factors affecting microspore developmental fate (Jain et al., 1996-1997). Genotype plays a major role in androgenesis, with important differences in response observed between species but also within cultivars of the same species (Jain et al., 1996-1997; Maluszynski et al., 2003; Seguí-Simarro and Nuez, 2008a; Ochatt et al., 2009; Germanà, 2011). The pollen developmental stage is a complex factor that has a large bearing on the success of androgenesis (Germanà, 2011). Optimal responses are normally obtained from the early uni-nucleate to the early bi-nucleate stage (Sangwan and Sangwan-Norreel, 1996). Microphenological traits such as bud length and anther size can be used as indicators of the microspore developmental stage. For pea, uni-nucleate microspores appear to be the most appropriate stage for initiation of haploid cultures (Croser et al., 2006; Ochatt et al., 2009; Lülsdorf et al., 2011). The uni-nucleate stage corresponds to a flower bud length of 6 to 7 mm and an anther size of 1 mm.

There is no clear consensus in the literature on the specific culture conditions required for doubled haploidy of grain legumes (Croser et al., 2006; Lülsdorf et al., 2011). However, key factors that require optimisation in the development of every protocol have been identified. Some of these factors include light conditions (i.e. light intensity, light quality and photoperiod), culture temperature, microspore culture density, medium composition for embryo induction, maturation and regeneration (i.e. macro and micro salt composition, plant growth regulators, osmolarity, pH, carbohydrate source) (Jain et al., 1996-1997; Croser et al., 2006; Ochatt et al., 2009;
The first report of haploid callus induction in pea was by Gupta et al. in 1972, with the later recovery of a few plants (Gupta, 1975), however, the haploid condition of these plants was not confirmed and the results were not reproduced. More recently, a cold pre-treatment of pea buds successfully induced symmetrical divisions, callus formation and early-stage embryo development from extracted anthers (Gosal and Bajaj, 1988; Sidhu and Davies, 2005; Bobkov, 2010) and isolated microspores (Croser and Lülsdorf, 2004; Croser et al., 2005). In 2009, Ochatt et al. were the first to recover a small number of flow cytometry-confirmed haploid plants from isolated microspores of pea cultivars Frisson, CDC April and Victor. However, these plants were weak and did not survive greenhouse transfer. The combined application of multiple stress treatments viz. cold, electroporation and osmotic shock, was critical to the successful elicitation of haploid divisions and subsequent haploid embryogenesis. Also, the initial microspore culture density had a significant effect on microspore division rate. A density of $2 \times 10^5$ microspores/ml provided the most consistent responses. On the other hand, other culture factors such as temperature or light conditions, as well as medium basal salts and hormonal composition had little influence on androgenetic responses in pea. It is clear the DH protocols published to date are not robust enough to be used in a pea breeding program and have been applicable to a very limited number of cultivars.

A wide variety of stress treatments have been successfully applied across species for the reprogramming of microspores towards haploid embryogenesis (Shariatpanahi et al., 2006). A review of the literature has shown cold pre-treatment
of buds, electroporation of anthers or isolated microspores, stepwise osmotic modification of the induction medium and centrifugation of microspores appear to be the most effective for legumes. The positive effect on androgenesis of a bud/ anther cold pre-treatment has been reported for the legumes pigeonpea (*Cajanus cajan* L.) (Kaur and Bhalla, 1998), chickpea (*Cicer arietinum* L.) (Grewal et al., 2009) and pea (Gosal and Bajaj, 1988; Croser et al., 2005; Ochatt et al., 2009). Electroporation of isolated microspores improved regeneration competence in *Asparagus officinalis* L., canola (*Brassica napus* L.), tobacco (*Nicotiana tabacum* L.), chickpea, pea, *Lathyrus* spp. and *Medicago truncatula* Gaernt. (Mishra et al., 1987; Jardinaud et al., 1993; Delaitre et al., 2001; Grewal et al., 2009; Ochatt et al., 2009). Modification of osmotic pressure was essential for the continued development of induced microspores of chickpea, pea, *Lathyrus* spp. and *M. truncatula* (Croser et al., 2006; Grewal et al., 2009; Ochatt et al., 2009). In pea, an osmotic stress treatment has been shown to improve callusing of microspores by favouring a slight detachment of membranes from the microspore wall and thus facilitating initial divisions (Ochatt et al., 2009). A centrifugal shock has been used for the successful induction of androgenesis in chickpea (Grewal et al., 2009). Centrifugation of chickpea anthers at 168 g for 10 min resulted in early embryo formation and a greater proportion of embryos per anther compared to untreated anthers. More recently, the addition of n-butanol in the induction medium has been reported to stimulate androgenesis in wheat (*Triticum aestivum* L.) (Soriano et al., 2008; Broughton, 2011). It has been proposed that the increase in number of haploid embryos and plants may be due to the disruption of cortical microtubules by n-butanol (Soriano et al., 2008). Unpublished results from our laboratory at UWA indicated that n-butanol also effectively enhances the switch from gametic to sporophytic development in chickpea (Croser pers. comm. 2010). I have
therefore selected these factors as the basis for my initial experiments to improve robustness of the DH process for pea.

The aim of the research presented in this chapter was to use the existing body of literature to develop a protocol for the regeneration of haploid plants from cultured anthers of pea. I reason that the application of key stress factors and the optimisation of the culture medium and culture conditions will lead to a robust in vitro protocol for the routine induction of sporophytic development from extracted anthers in a range of pea cultivars.
2.2 Materials and methods

2.2.1 Plant material

A range of genetically diverse pea (*Pisum sativum* L.) genotypes were selected for this research, *viz.* the Australian cultivars Kaspa, Mukta, Dunwa, Helena, Excell and Bundi; the French cv. Frisson; and the Canadian cv. CDC April (Table 2.1).

**Table 2.1** Pea cultivars used for the anther culture protocol development

<table>
<thead>
<tr>
<th>Seed type*</th>
<th>Kaspa</th>
<th>Mukta</th>
<th>Dunwa</th>
<th>Helena</th>
<th>Excell</th>
<th>Bundi</th>
<th>Frisson</th>
<th>CDC April</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dun, round</td>
<td>White, round</td>
<td>Dun, dimpled</td>
<td>Blue, round</td>
<td>White, round</td>
<td>White, round</td>
<td>Dun, dimpled</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dun, round</td>
<td>White, square</td>
<td>Dun, dimpled</td>
<td>Blue, round</td>
<td>White, round</td>
<td>White, round</td>
<td>Dun, dimpled</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seed type**</td>
<td>SL (af)</td>
<td>SL (af)</td>
<td>C (Af)</td>
<td>C (Af)</td>
<td>SL (af)</td>
<td>SL (af)</td>
<td>C (Af)</td>
<td>SL (af)</td>
</tr>
<tr>
<td>Leaf type**</td>
<td>Late</td>
<td>Late</td>
<td>Mid/Late</td>
<td>Mid</td>
<td>Early/Mid</td>
<td>Early</td>
<td>Early</td>
<td>Late</td>
</tr>
<tr>
<td>Flower colour</td>
<td>Pink</td>
<td>White</td>
<td>Purple</td>
<td>Purple</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>Purple</td>
</tr>
<tr>
<td>Flowering/Maturity</td>
<td>Late</td>
<td>Late</td>
<td>Mid/Late</td>
<td>Mid</td>
<td>Early/Mid</td>
<td>Early</td>
<td>Early</td>
<td>Late</td>
</tr>
<tr>
<td>Plant height***</td>
<td>SD (le)</td>
<td>SD (le)</td>
<td>T (Le)</td>
<td>T (Le)</td>
<td>SD (le)</td>
<td>SD (le)</td>
<td>SD (le)</td>
<td>SD (le)</td>
</tr>
</tbody>
</table>

*SL: semi-leafless type; C: conventional leaf type; T: tall type; SD: semi-dwarf type.

*The Dun type is variously dimpled with greenish-brown testa and yellow cotyledons (Khan and Croser, 2004).

**In the recessive mutants designated as afila (af), leaflets are modified into tendrils. Plants with afila characteristics are popularly known as semi-leafless (Khan and Croser, 2004).

***Le: internode length locus. The principal effect of *le* is to reduce the length of the upper internodes and cause a zig-zag appearance of the stem (semi-dwarf type) (Reid et al., 1983).

2.2.2 Donor plant growth conditions

Seeds were sown in a glasshouse in 180 mm pots filled with potting mix (UWA Plant Bio Mix – Richgro Garden Products Australia Pty. Ltd.) (Fig. 2.1). The temperature was controlled at 20°C day/ 18°C night under natural light conditions (lat: 31°58’49” S; long: 115°49’7” E). Plants were watered daily and fertilised fortnightly with a water soluble N-P-K (19-8.3-15.8) fertiliser with micronutrients (Poly-feed, Greenhouse Grade, Haifa Chemicals Ltd.) at a rate of 3 g/pot.

**Figure 2.1** Glasshouse grown pea donor plants (cvs. Frisson and Kaspa).
2.2.3 Harvest of buds and determination of microspore developmental stage

To confirm the correlation of the bud size to the uni-nucleate stage of microsporogenesis, flower buds at various sizes (4-4.9, 5-5.9, 6-6.9 mm of length) were harvested daily for each of the genotypes studied and the developmental stage of microspores immediately determined by DAPI (4’-6’-Diamidino-2-phenylindole.2HCl) stain. Buds of various sizes were opened and the anthers contained within were placed in 1.5 ml centrifuge tubes containing 500 µl of Carnoy’s fixative (3: 1 90% Ethanol: Glacial Acetic Acid) and stored at 4°C for 24 h for fixation. After 24 h, the Carnoy’s fixative was carefully removed using a glass pipette. One anther per treatment was then placed on a slide and 20 µl of a freshly prepared DAPI (Sigma D-8417) working solution (25 µl of 1 mg/ml DAPI stock solution + 1 ml of 0.3 M mannitol) was added. Anthers were quickly dissected in this solution to release the microspores, anther debris was removed and a coverslip applied. Samples were then incubated in the dark for at least 30 min at room temperature and the development of at least 100 microspores per treatment was examined with a Zeiss fluorescent microscope (Carl Zeiss, Germany) fitted with DAPI wavelength appropriate filter set. Nuclei were stained bright blue. Images were obtained using an Axiocam camera and annotated using AxioVision Imaging System software (Carl Zeiss, Germany).

2.2.4 Effect of bud harvest number on microspore viability

As an indeterminate species, the pea plants could be harvested many times. An experiment was established to determine the effect of multiple harvests on microspore viability. Buds of cv. Frisson containing microspores at the uni-nucleate stage were harvested sequentially six times and the microspore viability immediately determined by fluorescein diacetate (FDA) staining (Heslop-Harrison and Heslop-
Harrison, 1970). A minimum of 200 microspores per treatment were counted using a Zeiss fluorescence microscope (Carl Zeiss, Germany). Viability was determined as percentage of microspores fluorescing yellow-green under UV light. Images were obtained using an Axiocam camera and annotated using AxioVision Imaging System software (Carl Zeiss, Germany).

2.2.5 Bud sterilisation

Flower buds (approximately 100 buds) were placed in a 100 ml beaker and surface sterilised by treatment for 1 min in 70% v/v ethanol, followed by 10 min 1% v/v commercial sodium hypochlorite (White King bleach, Pental Products Pty Ltd, Australia). Buds were rinsed three times with sterile water for 1 min.

2.2.6 Culture media

Different media composition and plant growth regulators were used in order to induce haploid divisions. The media used in these experiments were HSO (Ochatt et al., 2009), NLN (Lichter, 1982) and JFK (see Appendix 1). These media were all tested with no hormones or with the addition of 2,4-Dichlorophenoxyacetic acid (2,4-D) (1mg/L), picloram (0.25 mg/L) and 6-Benzylaminopurine (6-BAP) (0.1mg/L). All media components were autoclaved and only plant growth regulators were filter sterilised.

2.2.7 Anther extraction procedure

Anthers were extracted using forceps and needles under sterile conditions using a dissecting microscope and making sure any remnants of filament were removed. Ten anthers per treatment were cultured into a 10 x 35 mm Petri dish containing 2 ml of liquid medium. Dishes were sealed with Parafilm® and incubated for four weeks at 24°C under dark or light conditions (90 μmol m⁻² s⁻¹ and a 16/8 h light/dark photoperiod).
2.2.8 Stress treatments

A series of experiments were undertaken to study the effect of the individual and combined application of stress treatment/s on the elicitation of androgenesis from extracted anthers of a range of pea genotypes (Table 2.1).

2.2.8.1 Cold pre-treatment

Flower buds containing microspores at the uni-nucleate stage were placed in sealed Petri dishes (approximately 30 buds per treatment) and stored under dry conditions in the fridge at 4°C in the dark for 0, 7, 14 and 21 days before culture. Microspore viability was determined after each cold pre-treatment, by fluorescein diacetate (FDA) staining (Heslop-Harrison and Heslop-Harrison, 1970) as described in Section 2.2.4. Cold pre-treated buds were then sterilised as described in Section 2.2.5. Anthers were extracted and directly cultured (see Section 2.2.7) or cultured after submission to the various combinations of stress treatments.

2.2.8.2 Electroporation

Thirty anthers were dispensed into a cuvette with electrodes 2 mm apart containing 1300 µL of Rech et al. (1987) electroporation buffer. Three successive exponential pulses of 0, 750, 1000 or 1500 V/cm with a capacitance of 0, 75, 100, 150 µF and a constant resistance of 50 Ω were delivered at 10 s intervals using an Electro Cell Manipulator ECM630 (BTX Gentronics Biomedical Ltd., San Diego, USA) (Fig. 2.2).

Figure 2.2 Electro cell manipulator used for the application of the electric shock treatment.
2.2.8.3 Centrifugation

Thirty intact anthers per treatment were placed in 10 ml glass tubes containing 8 ml of electroporation buffer (Rech et al., 1987) and centrifuged at 170 g for 10 min, at 4°C using a Jouan CR4-22 Benchtop Centrifuge (Jouan Inc, Winchester, VA, USA).

2.2.8.4 Sonication

Thirty intact anthers per treatment were sonicated in 10 ml glass tubes containing 8 ml of electroporation buffer (Rech et al., 1987) at 38 KHz for 30 s, at room temperature using a Branson 1210 Sonicator (Bransonic Ultrasonic Co, Danbury, CT, USA).

2.2.8.5 Osmotic shock

Anthers were plated into 35 x 10 mm Petri dishes (10 anthers/dish) with 2 ml of NLN, HSO or JFK medium containing 17% (w/v) of sucrose. After 7 days of culture, anthers were transferred to the same medium containing 10% (w/v) of sucrose (Ochatt et al., 2009).

2.2.8.6 n-butanol treatment

Thirty anthers of cv. Frisson per n-butanol treatment were extracted and cultured in Petri dishes (10 anthers per dish) containing 2 ml of hormone-free HSO medium. Various concentrations (0, 0.1, 0.3 and 0.5%) of n-butanol (99.8% 1-butanol, anhydrous, Sigma-Aldrich™ Co.) were added to the medium for 5 h at D0, D2 and D5 of culture. During the treatments, dishes were covered with aluminium foil and left unsealed in the laminar flow cabinet (Broughton, 2011). After the treatment, the culture medium with the n-butanol was removed by pipette and 2 ml of the same fresh medium were added to each dish.
2.2.9 Embryo development

After four weeks of culture in liquid medium, at least 30 early embryoids per treatment were transferred to two alternative media sequences in order to support embryo development and germination and cultured as described in Section 2.2.7: 1- Media sequence devised by Lehminger-Mertens and Jacobsen (1989) (LMJ media); or 2- Media sequence designed by Loiseau et al. (1995).

**LMJ media sequence:** The basal medium consists of MS salts (Murashige and Skoog, 1962) + B5 vitamins (Gamborg et al., 1968) plus 2% sucrose, 5% mannitol, 0.1% (w/v) casein hydrolysate, 0.6% of agar and pH: 5.6. For embryo induction 2,4D (4 µM) is added to the basal medium. Embryos are then transferred to basal medium with no added hormones. Embryo maturation is promoted by culturing in a medium with GA₃ (1.5 µM) and NAA (0.12 µM). Finally, embryo germination is obtained by adding GA₃ (2.9 µM) to the basal medium. Embryo transfer from one medium to another was done based on the visual assessment of the developing embryos, as per Ochatt et al., 2002.

**Loiseau et al. media sequence:** The composition of the basal medium is: MS salts (Murashige and Skoog, 1962) + B5 vitamins (Gamborg et al., 1968) plus 0.1% (w/v) casein hydrolysate, 0.7% agar, 9% sucrose and pH: 5.6. Calli are cultured for 4 weeks in 90 mm Petri plates containing 50 ml of the basal medium with 4.5 µM of 2,4-D. After that period, calli/embryos are transferred to the same medium with no hormones.

2.2.10 Tracking experimental results

Microspore embryo development was checked every 48 h from culture using an Olympus CK2 inverted microscope (Olympus Optical Co. Ltd., Japan). In case of observation of embryo development/callusing, two anthers per treatment were
sampled and split open on a slide, and a few drops of 2% acetocarmine stain were added. Samples were then observed under 40x magnification (light microscope) in order to detect if microspores within anthers were developing. Also, DAPI-staining was used to track microspore development within anthers during culture: two anthers per treatment were collected at D0, D7 and D14 and DAPI-stained and analysed as previously described in Section 2.2.3.

The experimental design was completely randomised and all treatments were repeated at least three times with a minimum of 10 replicates per treatment per genotype. Statistical analysis was performed by analysis of variance (Microsoft Office Excel 2007 software).
2.3 Results

2.3.1 Correlation between pea bud length and microspore developmental stage

A correlation between bud size and microspore developmental stage was observed in most of the pea cultivars studied. For cultivars Frisson, Kaspa, Dunwa, Helena, Excell, Bundi and Mukta, flower buds 6-7 mm long contained microspores predominantly at the optimum, uni-nucleated stage (Fig. 2.3). However, in cv. CDC April, 4-5 mm long flower buds contained mainly microspores at the uni-nucleate stage. This was the optimal stage for induction of androgenesis divisions.

![Figure 2.3 Determination of microspore developmental stage. Pea flower buds containing anthers with microspores predominantly at the uni-nucleate stage (A and B). DAPI-stained microspores at the optimum uni-nucleated stage for androgenesis induction (C) and at a later, non inductive, stage (D), after first asymmetric mitosis.](image)

2.3.2 Effect of bud harvesting procedure and cold pre-treatment duration on microspore viability

The number of times each donor plant was harvested (up to the 6th harvest) had no significant effect on microspore viability at the day of harvesting for cv. Frisson (Fig. 2.4).
Figure 2.4 Microspore viability estimated by fluorescein diacetate (FDA). Analysis of the effect of harvest number on microspore viability at D0 in cv. Frisson (results are expressed as mean ± SD).

The cold pre-treatment of buds for up to 7 days did not significantly affect the microspore viability rate (Fig. 2.5). However, storing buds in the cold for more than 7 days significantly reduced the viability rate compared to the control treatment (0 days in cold). A 14 day cold pre-treatment reduced microspore viability to less than 60% while a 21 d pre-treatment further reduced it to less than 30%.

Figure 2.5 Microspore viability estimated by fluorescein diacetate (FDA). Analysis of the effect of bud cold pre-treatment duration on microspore viability in cv. Frisson (results are expressed as mean ± SD; P = 0.05).
2.3.3 Effect of culture medium plant growth regulators on androgenesis induction

Continuous culture of anthers in JFK medium, containing 2,4-D (1 mg/l), Picloram (0.25 mg/l) and BAP (0.1 mg/l) for 4 weeks led to the induction of androgenesis with consistent callus production. A transversal cut showed callus production from the microspores contained within the anthers rather than from the anther wall tissue (Fig 2.6). No callusing was observed when culturing anthers in hormone-free HSO and NLN media regardless of the stress treatment applied.

Figure 2.6 Transversal cut of cultured anther showing callus production from microspores within the anther (cv. Frisson).

2.3.4 Effect of culture conditions on callusing frequency

The effect of culture light conditions (light vs. dark) and electroporation field strength (0, 500 and 1000 V/cm) on callusing response from extracted anthers of cvs. Frisson and CDC April was studied. In both genotypes, the highest percentage of callusing was obtained by culturing anthers for the first four weeks under dark conditions, disregarding the electroporation treatment applied. No androgenetic response was obtained for cv. CDC April when cultured under light (90 µmol m⁻² s⁻¹) (Fig. 2.7).
2.3.5 Effect of individual and combined stress treatments on elicitation of androgenesis

A number of eliciting factors were studied for their effect on androgenetic competence of pea. The application of multiple stress treatments induced symmetrical divisions of the nuclei leading to the development of multinucleate syngtiums (Fig. 2.8A and 2.8B) and callus production (Fig. 2.8C). As a result of these treatments, an enlargement of microspore/ anther size (hyperplasia) was observed after androgenesis induction (Fig. 2.9).
Figure 2.8 DAPI-stained pea microspores showing symmetrical division of the nucleus (A) and multinucleate syngamy (B). Inverted microscope image showing pea microspore derived callus production (C).

Figure 2.9 Initial androgenetic response observed in cultured anthers at D7 of culture (cv. Frisson). Response obtained after a cold pre-treatment of buds combined with centrifugation and electroporation of intact anthers. A) Unresponsive and responsive (hyperplasia) anthers; B) Embryoid development from intact anther.

Electroporation of anthers containing microspores at the uni-nucleate stage at electrical parameters optimised for each genotype (Fig. 2.7, Fig. 2.10 and Fig. 2.11) promoted the best callusing responses for the genotypes studied. Centrifugation and/or sonication alone did not suffice to induce androgenesis. For cv. CDC April best
responses were obtained with anther electroporation at 1000 V/cm and 100 µF of capacitance, while 500 or 1000 V/cm and 75 or 100 µF were best for cv. Frisson.

**Figure 2.10** Effect of electroporation voltage on percentage of callusing from anthers of pea cvs. Frisson and CDC April at optimum developmental stage (results are expressed as mean ± SD; P = 0.05).

The combined application of multiple stress treatments, including a cold pre-treatment, electroporation, centrifugation and sonication of anthers prior to culture and an osmotic shock during the first week of culture, promoted androgenetic proliferation and subsequent differentiation from microspores within anthers of the
pea genotypes studied (Fig. 2.12). On the other hand, none of the n-butanol stress treatments applied successfully induced haploid divisions from cultured anthers (data not shown).

Figure 2.12 Effect of different combinations of stress treatments on percentage of callusing after 28 days of culture from extracted anthers of cv. Kaspa (O: osmolarity; S: sonication; C: centrifugation; E: electroporation). In all treatments buds were previously submitted to a cold treatment for 7 d at 4°C. Results are expressed as mean ± SD; P=0.05.

2.3.6 Embryo maturation and germination

After four weeks of culture in JFK medium, the calli obtained (friable and yellow-green in colour) were transferred to the media sequence devised by Lehminger-Mertens and Jacobsen (1989) or to the media sequence devised by Loiseau et al. (1995). No further development was achieved when culturing using the LMJ media sequence. On the other hand, the culture of calli in the Loiseau media sequence led to the production of advanced stage anther-derived embryos (heart-shaped and cotyledonary stages) in cvs. Frisson, Kaspa, Helena and Bundi (Fig 2.13). However, using this protocol, no haploid plantlet germination was achieved for any of the pea cultivars studied.
Figure 2.13 Anther culture derived somatic embryos of pea cv. Frisson. Arrow colour indicates different embryo developmental stage (white: pro-embryo; red: globular; blue: heart; black: torpedo).
2.4 Discussion

In the research presented in this Chapter, a series of key factors in the development of a robust pea anther culture protocol were identified. The combined application of multiple stress treatments and the optimisation of key culture factors led to the development of an efficient protocol for the routine induction of androgenesis from extracted anthers of a range of pea genotypes. Advanced-stage embryos were consistently achieved; however, no haploid plants were recovered with the strategy proposed. Further research is required before a robust and reliable method for doubled haploid plant production across a range of pea genotypes is available.

Anther culture conditions (i.e. temperature and light conditions), microspore developmental stage and the combined application of multiple stress treatments were identified as critical factors for the induction of androgenic division in pea anther culture. Induction of sporophytic development was achieved in uni-nucleate microspores within anthers of pea via the application of a cold (4°C) pre-treatment to buds for 2 to 14 days combined with electroporation, centrifugation and sonication treatments before culture and an osmotic shock during the first 7 d of culture. These results are consistent with those published by Ochatt et al. (2009) for isolated microspore culture of pea. In addition, sonication is reported as a novel abiotic stress factor for the elicitation of androgenesis in pea. On the other hand, the n-butanol stress treatment failed to induce androgenic divisions from cultured anthers. Microscope analysis during culture showed symmetrical division of the nuclei was followed by the development of multinucleate syncitials, callus formation and somatic embryo development. With the proposed protocol, torpedo-shaped embryos
were routinely achieved from extracted anthers of pea but no haploid plantlets were recovered.

Little is known about the exact mechanisms underlying the elicitation of androgenesis using stress treatments. However, attempts have been made to explain the possible effects of some of these stress factors. It has been proposed that a cold pre-treatment slows-down the degradation process in the anther tissue thus protecting microspores from toxic compounds released from the decaying anthers (Duncan and Heberle-Bors, 1976) and favours the synchronisation of the microspore developmental stage (Hu and Kasha, 1999). Also, a cold pre-treatment appears to increase the frequency of endo-reduplication during androgenesis induction (Amssa et al., 1980), a phenomenon that has long been suggested as a mechanism to explain the occurrence of higher DNA content in induced microspores (Seguí-Simarro and Nuez, 2008b). In fact, Raquin et al. (1982) observed in wheat that a high proportion of uni-nucleate microspores at G2-phase (2C DNA content) increased their DNA content to 4C after cold induction while maintaining their uni-nucleate status. Electric pulses have been shown to modify the structure of the plasma membrane, presumably by the temporary formation of pore-like openings which facilitate the uptake of media components into the cells (Neumann and Rosenheck, 1972; Zimmermann et al., 1976; Kinosita and Tsong, 1977; Ochatt, 2013). Electroporation has also been shown to stimulate DNA synthesis in cultured plant protoplasts (Rech et al., 1988). An accumulation of ABA has been observed in osmotic stressed anthers of tobacco (Imamura and Harada, 1980) and barley (*Hordeum vulgare* L.) (Van Berger et al., 1999), which may be involved in the reprogramming of microspore development (Wang et al., 2000; Žur et al., 2008).
Changes in endogenous hormone levels following stress treatments has been observed in a number of species during androgenesis elicitation (Wang et al., 2000; Seguí-Simarro and Nuez, 2008a; Żur et al., 2008). In pea, Lülsdorf et al. (2012) observed important hormonal changes during androgenesis induction from stress-treated anthers. They primarily detected an increase in active auxins (mainly IAA-asp) after the application of individual and combined stress treatments. In the research presented in this chapter, the addition of plant growth regulators to the culture medium promoted callus proliferation from microspores of pea within anthers. Transversal cut of cultured anthers showed that callus was originating from dividing microspores within the anthers rather than from the diploid maternal tissue. However, there is a possibility that some of the callus tissue obtained may have originated from the anther wall tissue. From these results it is not possible to infer if exogenous hormones are involved in the elicitation androgenesis from cultured anthers of pea or if they have an effect in promoting callusing after stress treatment induction. For that reason, it will be of great value to identify a strategy to assess the effect of individual and combined stress treatments on the elicitation of androgenesis.

The development of a robust pea DH protocol will facilitate the rapid establishment of useful traits and the released of improved cultivars. This will permit a rapid response to changing conditions. However, despite the significant effort to date, there is no universal DH protocol effective in every species and success remains highly genotype-dependent (Ochatt et al., 2009; Wędzony et al., 2009). Legume species in particular, are still regarded as recalcitrant to this technology (Croser et al., 2006; Lülsdorf et al., 2011). The main difficulty in improving existing DH protocols lies in the great variety of factors that influence and control androgenesis (Wędzony et al.,
Much of the research work in DH protocol development is limited to the identification of responsive genotypes, the optimisation of culture conditions and the application of a variety of stress treatments, while the understanding of the fundamental mechanisms involved in haploid induction and the decisive role of stress treatments remains elusive.

From the results presented in this chapter I could identify and optimise some of the key factors involved in haploid embryogenesis from cultured anthers of pea. Advanced-stage embryos were consistently achieved but no haploid plants were recovered with the proposed strategy. The application of multiple stress treatments was clearly crucial for the induction of haploid divisions from extracted anthers of pea. The focus of the following chapter will be a flow cytometry study of the effect of these stress treatments on the elicitation of androgenesis from intact anthers and isolated microspores of pea.
2.5 References


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Chapter 3

Flow cytometry enables identification of sporophytic eliciting stress treatments in gametic cells

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3.1 Introduction

Flow cytometry is a method to rapidly and reproducibly determine relative nuclear DNA content and ploidy level of cellular samples. Flow cytometry has been widely applied for the analysis of animal cells (Curran, 1999) and more recently, plant cells (Doležel et al., 2007a) following the advent of simple and reproducible methods for tissue analysis (Galbraith et al., 1983; Doležel et al., 2007b). Applications to plant cell analysis include, but are not limited to, differentiation between cells within a mixed cell population, analysis of chemical composition of different tissues, measurement of secondary metabolite accumulation and ploidy determination following haplo-diploidisation (Yanpaisan et al., 1999; Doležel et al., 2007a; Ochatt, 2008). Despite this, flow cytometry has been applied on a relatively limited scale for analysis of embryogenic potential in plant cell populations (Ochatt et al., 2000; Ochatt, 2008; Ochatt et al., 2011).

Flow cytometry analysis has shown a correlation between light-scatter and fluorescence properties of cultured cells and their embryogenic potential. In 2000, Ochatt et al. proposed flow cytometry as a tool for early prediction of plant
regeneration competence from protoplasts of pea (*Pisum sativum* L.). Fertile plants were produced only from calli with a normal relative DNA content profile. Schulze and Pauls (2002) developed a flow cytometry method to assess embryogenic potential of cultured microspores of rapeseed (*Brassica napus* L.). By staining with calcofluor white the authors were able to follow synthesis of cellulose in microspores, thought to be an early indicator of embryogenic potential. Later, Ochatt (2008) also showed the importance of relative nuclear DNA content to assess embryogenic competence from calli of *Medicago truncatula* Gaertn. On the other hand, cell wall thickness of microspores was also shown to be a reliable indicator of embryogenesis competence in legumes by Ochatt et al. (2008). However, to our knowledge, the use of flow cytometry to study androgenic potential of microspores of any plant species by analysing their relative nuclear DNA content has not been reported.

Stress treatments have been widely adopted to induce the switch from gametophytic to sporophytic development for induction of microspore embryogenesis (Jain et al., 1996-1997; Touraev et al., 1997; Shariatpanahi et al., 2006). There is a large variety of proposed stress treatments, with a recent review by Shariatpanahi et al. (2006) listing 16 different stresses, which did not include the novel stress of sonication proposed within this report. However, until now, there has been no simple tool for assessing the actual effect of these stress treatments on subsequent DNA synthesis.

For legume species, pea, chickpea (*Cicer arietinum* L.), *M. truncatula* and grass pea (*Lathyrus spp.*), we have demonstrated pyramiding (meaning successively superimposed) multiple stress treatments *viz.* cold treatment, electroporation and osmotic stress, is critical to successful elicitation of haploid division and subsequent
haploid embryogenesis* (Grewal et al., 2009; Ochatt et al., 2009). Cold pre-treatment has been shown to synchronise microspore development and foster microspore division in a number of species (Jain et al., 1996-1997; Touraev et al., 1997; Delaitre et al., 2001; Maluszynski et al., 2003), including legumes (Kaur and Bhalla, 1998; Croser et al., 2006; Skrzypek et al., 2008; Grewal et al., 2009; Ochatt et al., 2009). Electroporation has been shown to stimulate DNA synthesis (Rech et al., 1988) and improve regeneration competence from protoplasts of various species (Rech et al., 1987; Ochatt et al., 1988). Electroporation is an effective stress treatment for induction of haploid embryogenesis from microspores of tobacco (*Nicotiana tabacum* L.) (Mishra et al., 1987), rapeseed (Jardinaud et al., 1993), *Ginkgo biloba* L. (Laurain et al., 1993), *Asparagus officinalis* L. (Delaitre et al., 2001), and legumes (Grewal et al., 2009; Ochatt et al., 2009). Improved androgenic response after modification of osmotic pressure has been observed in a number of species (Jain et al., 1996-1997; Lionneton et al., 2001), including legumes (Croser et al., 2006; Grewal et al., 2009; Ochatt et al., 2009). Centrifugation is applied as part of many microspore isolation protocols, but has also been used as a stress treatment for induction of androgenesis from intact anther culture in chickpea (Grewal et al., 2009), tobacco (Tanaka, 1973), *Datura innoxia* Mill. (Sangwan-Norreel, 1977) and rapeseed (Aslam et al., 1990). However, the need for combining stress treatments for the induction of androgenesis appears to be confined to species of the *Fabaceae*.

Observations from our previous research led us to hypothesize stress treatments may be categorized as either elicitors or enhancers of androgenesis. We consider as eliciting those stress treatments required to induce the developmental shift from gametophytic to sporophytic pathway. Enhancing treatments, whilst not

* Refer to Chapter 2 – P.64
eliciting, improve androgenetic embryogenesis after elicitacion. To test this, we used flow cytometry to assess the effect of individual and combined stress treatments on relative nuclear DNA content of microspores.
3.2 Materials and methods

3.2.1 Plant material

Pea (*Pisum sativum* L.) cv. Frisson and the F<sub>1</sub> from a cross cv. Frisson x cv. Cameor were grown in a glasshouse at 20/18°C, with a 16/8 h light/dark regime of 220 µE/ m<sup>2</sup> s from 400 W sodium lamps. Pots were filled with volcanic rock (pozzolanne). Plants were watered and nourished by capillarity with the standard nutrient solution used in INRA greenhouses: 14.4 mM NO<sub>3</sub>, 3.94 mM NH<sub>4</sub>, 15.8 mM Ca, 17.9 mM K<sub>2</sub>O, 4 mM MgO, 2.46 mM P<sub>2</sub>O<sub>5</sub>, 2 mM SO<sub>3</sub> and various micro-elements. Flower buds 7 mm long containing anthers 1 mm in length and with microspores predominantly at the uninucleate stage, optimal for induction of microsporogenesis (Fig. 3.1), were harvested and subjected to a cold treatment at 4°C in the dark for 7 to 21 d.

![Figure 3.1](image)

**Figure 3.1** Determination of microspore developmental stage. Cytological development of male gametophyte in pea. DAPI-stained microspores showing uninucleated (A), bi-nucleated (B) and multi-nucleated stages (C and D). Flower buds and anthers at the optimal developmental stage for androgenesis (E, F and G).

3.2.2 Bud sterilization and microspore isolation

Flower buds were placed in a 100 ml beaker and surface sterilized by treatment for 1 min in 70% v/v ethanol, followed by 10 min in calcium hypochlorite (70 gL<sup>-1</sup>). Buds were rinsed three times with sterile water (refer to Chapter 2, Section 2.2.5).
Buds were transferred to a sterile mortar and macerated with a pestle in electroporation buffer (Rech et al., 1987). Three successive steps of suspension and centrifugation (100 g, 10°C, 5 min each) were used to eliminate debris remaining after serial sieving through 80, 60 and 40 µm filters (Ochatt et al., 2009).

Density was determined using a Fuchs Rosenthal haemocytometer and adjusted for culturing to $1 \times 10^5$ microspores/ ml or, for electro-stimulation and sonication treatments, to $1 \times 10^6$ in electroporation buffer.

### 3.2.3 Stress treatments studied to elicit and/or enhance androgenesis

Extracted anthers and isolated microspores were submitted to the following stress treatments either individually or in combination: cold, electroporation, sonication, centrifugation and osmotic shock. The relative nuclear DNA content of extracted anthers and isolated microspores was immediately analyzed using a Partec PA II flow cytometer or analyzed after 7 d and 14 d of culture. All flow cytometry assessments were repeated at least three times over a period of two months and a minimum of 2500 nuclei per run were counted.

#### 3.2.3.1 Electroporation

Isolated microspores and extracted anthers were dispensed into cuvettes of an Electro Cell Manipulator ECM630 (BTX Gentronics Biomedical Ltd., San Diego, CA, USA), containing electroporation buffer (Rech et al., 1987). For electroporation of isolated microspores, a 600 µL aliquot of buffer containing $6 \times 10^5$ microspores was dispensed into a cuvette with electrodes spaced 1 mm apart. For electroporation of anthers, a 1300 µL aliquot of buffer was dispensed into a cuvette with electrodes spaced 2 mm apart and 30 anthers were added. Three successive exponential pulses
of 1500 V/cm (anthers) or 750 V/cm (anthers and microspores) with a capacitance of 100 µF and a constant resistance of 50 Ω were delivered at 10 s intervals.

3.2.3.2 Centrifugation of extracted anthers

Thirty intact anthers per treatment in 8 mL of electroporation buffer were centrifuged with brakes off in 10 mL glass tubes at 170 g for 10 min, at 4°C using a Jouan CR422 centrifuge. For microspores, centrifugation was applied during isolation.

3.2.3.3 Sonication of extracted anthers and isolated microspores

Thirty intact anthers per treatment in 8 mL of electroporation buffer and 1x 105 microspores per treatment in 2 mL of the same buffer were sonicated in 10 mL glass tubes at 38 kHz for 30 s, at room temperature using a Branson 1210 sonicator (Branson Ultrasonic Corporation, USA).

3.2.4 Flow cytometry analysis

For flow cytometry analysis, a Partec PA-II flow cytometer equipped with a HBO-100 W mercury lamp and a dichroic mirror (TK420) was used. The relative nuclear DNA content of 1) extracted anthers and 2) isolated microspores of pea, after submission to various stress treatments and times of culture, was compared with leaf controls from seed-derived plants of the two genotypes studied. In addition, the cell-division cycle was analyzed and mitotic index calculated (Doležel et al., 2007a; Ochatt, 2008).

Nuclei were mechanically isolated by chopping tissues* with a razor blade in 2 mL of single-step isolation + stain buffer (Partec®) containing 4,6 diamidino-2-phenylindole (DAPI), and the suspension was filtered through a 50 µm nylon mesh (Galbraith et al., 1983; Elmaghrabi and Ochatt, 2006; Doležel et al., 2007b). The collected data were analyzed with Partec Flomax software (Partec®, Partec GmbH, *See Appendix 2.
Germany). The key feature of DNA probes is that they are stoichiometric, and hence the number of molecules of the probe bound is equivalent to the number of molecules of DNA that are present. The excitation light intercepts the stained particle, which then emits a fluorescence signal that is transferred via optical system of the flow cytometer to photomultipliers. This signal is then converted from an analog pulse waveform to a corresponding digital value and processed by the computer to produce a one-parameter or two parameter dot-plot histogram.

3.2.5 Fluorescein diacetate viability test

The viability of extracted anthers and isolated microspores following stress treatments was determined by fluorescein diacetate (FDA) staining (Heslop-Harrison and Heslop-Harrison, 1970), after counting a minimum of 200 microspores per treatment using a Leika TMD fluorescence microscope (B1 IF 420-485 filter). Viability was determined as percentage of microspores fluorescing yellow-green under UV light. Images were obtained using a 3CCD Sony PowerHAD camera and annotated using Archimed Pro (Microvision France) software, as detailed elsewhere (Ochatt et al., 2008; Ochatt and Moessner, 2010).

3.2.6 Culture conditions

Pre-treated anthers and isolated microspores* were plated into 35 x 10 mm Petri dishes with 2 mL of NLN medium (Lichter, 1982) containing 17% (w/v) of sucrose. After 7 d of culture samples were transferred to the same medium containing 10% (w/v) of sucrose (Ochatt et al., 2009)**. The culture conditions were as follows: 22/19°C, with a 16/8 h light/dark regime of 90 µE/ m² s from warm white fluorescent tubes (Osram L58W/245 Universal White).

*Refer to Section 3.2.3; ** Refer to Chapter 2, Section 2.2.8.5.
3.3 Results

3.3.1 Flow cytometry analysis of extracted anthers

Differences in relative nuclear DNA content of microspores within anthers after stress treatment/s were clearly evident from analysis of the flow cytometry profiles. The profiles obtained from a diploid, glasshouse-grown leaf sample (Fig. 3.2A) were compared with those obtained from anthers submitted to stress treatment/s and then cultured for 0, 7 and 14 d. A normal (euploid) profile consists of two peaks, corresponding to nuclei in G1 phase of mitosis (2C DNA) and those in G2/M (4C DNA). Depending on treatment, profiles showed either two peaks (2C and 4C ploidy level) or three peaks (1C, 2C and 4C ploidy level). The results were analyzed in terms of presence/absence of haploid peaks in the profiles, coupled with their intensity (relative position) and magnitude (expressed by the percentage of the total population of nuclei analyzed they represent).

Pre-treating anthers at 4°C and transferring from a high to low osmolarity medium at day 7 of culture did not induce DNA synthesis as only 2C and 4C peaks could be observed after 14 d of culture (Fig. 3.2B). The application of a cold pre-treatment, an osmotic treatment plus centrifugation and/ or sonication treatments also did not induce androgenesis (Fig. 3.2C and 3.2D). However, adding an electroporation treatment to cold stressed anthers prior to their culture and an osmotic stress treatment successfully induced DNA synthesis and enhanced androgenetic proliferation. This was evidenced by a flow cytometry profile with three peaks (1C, 2C and 4C) showing clear indication of androgenic divisions within microspores (Fig. 3.2E). The combination of these three treatments was therefore considered to elicit androgenesis.
In an effort to further improve microspore induction, we added a centrifugation and/or a sonication treatment to anthers subjected to cold, electroporation and osmotic stresses. There was a clear synergistic and additive enhancing effect on androgenesis induction after successively imposing these multiple stress factors (Fig. 3.2F).

Figure 3.2 Effect of stress treatments on relative DNA content per nucleus of pea microspores within anthers after 14 d of culture. C-DNA profiles showing 1C, 2C and 4C ploidy levels. (A) Field pea leaves; (B) Cold + Osmolarity treatments; (C) Cold + Osmolarity + Sonication treatments; (D) Cold + Osmolarity + Centrifugation treatments; (E) Cold + Osmolarity + Electroporation treatments; (F) Cold + Osmolarity + Electroporation + Centrifugation + Sonication treatments. Note in figure 3.2A the scale of the vertical axis is different to the other profiles as the data was obtained from leaf tissue.
Differences among treatments in terms of relative nuclear DNA content at 0 d (Fig. 3.3A) and 7 d (Fig. 3.3B) were not as evident as they were after 14 d of culture (Fig. 3.3C), indicative of a delayed improvement of responses due to stress treatments.

Figure 3.3 Flow cytometry profiles obtained immediately after submission to cold, electroporation, centrifugation and sonication stress treatments (A) or after 7 d (B) and 14 d (C) of culture.

Sonication treatment alone (Fig. 3.4A) or combined with other stress treatments (Fig. 3.4B and 3.4C) increased the production of tetraploid peaks, indicating a possible doubling effect of sonication treatment.

Figure 3.4 Flow cytometry profiles of cold pre-treated extracted anthers submitted to a sonication treatment alone (A) or combined with electroporation (B) or electroporation and sonication (C).

The analysis of relative nuclear DNA content of microspores was used to predict whether a combination of stresses were elicitors or enhancers of androgenesis. The relative nuclear DNA content of microspores was greater after submission to eliciting factors (cold, electro-stimulation and osmolarity) compared to enhancing factors (centrifugation and sonication), leading us to propose their roles as shown in Figure 3.5.
Figure 3.5 Pyramiding eliciting factors (red) and enhancing factors (blue) enhances androgenetic response from pea anthers.

3.3.2 Flow cytometry analysis of isolated microspores

Flow cytometry profiles obtained from a diploid, glasshouse-grown leaf sample (Figure 3.6A), were compared with those obtained from isolated microspores submitted to stress treatment/s and then cultured for 0, 7 and 14 d. All stress treatments presented profiles indicative of no spontaneous DNA doubling, with one haploid (1C) peak at day 0, 7 and 14 (Fig. 3.6B, 3.6C and 3.6D). There was no clear effect of stress treatments on DNA synthesis in isolated microspores.
Figure 3.6 Effect of treatments on relative nuclear DNA content of pea isolated microspores after 14 d of culture. (A) Pea leaves; (B) Cold + Osmolarity + Electroporation treatments; (C) Cold + Osmolarity + Sonication treatments; (D) Cold + Osmolarity + Centrifugation + Sonication treatments.

3.3.3 Microspore fluorescein diacetate viability test

Following the stress treatments, a very high (>85%) viability rate was observed for both isolated microspores or extracted anthers of pea, indicating that there was no immediate deleterious effect of stress treatments on viability (Fig. 3.7).

Figure 3.7 FDA viability test from isolated microspores of pea. HV: Electroporation (1500V/cm); HVS: Electroporation (1500V/cm) + Sonication; LV: Electroporation (750V/cm); LVS: Electroporation (750V/cm) + Sonication; S: Sonication.
3.4 Discussion

Flow cytometry analysis of relative nuclear DNA content of microspores within anthers has enabled us to divide stress treatments between two categories: a) elicitors or b) enhancers of androgenesis. Our previous research has clearly shown pyramiding stress treatments to be the key to sustained androgenic division from pea (Croser et al., 2006; Ochatt et al., 2009). This is the first report in a plant species to evaluate the effect of various stress treatments based on relative nuclear DNA content and to use this information to categorize them as ‘elicitors’ or ‘enhancers’.

The flow cytometry analysis showed that cold and electroporation stress treatment of androgenic tissue, coupled with an osmotic modification of the medium during culture had both an individual and an additive eliciting effect on androgenesis from pea microspores within anthers. Thus, these treatments are required in combination to induce the developmental shift from gametophytic to sporophytic pathway. Hence, these stress treatments act as “elicitors” of androgenesis. In addition, as observed previously (Grewal et al., 2009; Ochatt et al., 2009) there was a positive effect of pyramiding stress factors, including sonication and centrifugation, on the induction of embryo formation from intact anthers. Sonication and centrifugation, whilst not elicitors, improve androgenetic embryogenesis when combined with the eliciting stresses above and can therefore be described as “enhancing treatments”. The application of combined stress factors seems to be the way to overcome recalcitrance of legumes to androgenesis, likely mediated through increases in hormone levels in stressed anthers (Lülsdorf et al., 2011).

Whilst the mechanism of various more commonly applied stress treatments such as temperature (Duncan and Heberle, 1976; Amssa et al., 1980; Žur et al., 2008)
and osmotic regulation (Dunwell and Thurling, 1985; Ochatt et al., 2009) is relatively well understood, the effect of these stresses on DNA synthesis has been more difficult to determine. Previously, a cytological study using fluorochromes such as DAPI has been the method of choice for showing the effect of treatments on nuclei division (Ochatt et al., 2011). Whilst we are not advocating the flow cytometry analysis presented here as a replacement for this type of study, it will have a role to play in quickly ascertaining the effect of one or more stresses on a population. Flow cytometry analysis of relative nuclear DNA content of microspores should assist researchers in making choices about what stress or group of stresses are most effective in eliciting androgenic development, particularly during the process of protocol development, not only for legumes but also for other plant species.

The flow cytometry analysis presented here represents a relatively simple, quick and reliable way to analyze and discriminate the effect of various stress treatments on elicitation of androgenesis. Sample preparation requires a small amount of plant tissue (10-20 mg) and takes only a few minutes (approximately 2 min per sample) at a low cost per sample (less than $2 per sample). Analysis is rapid, and a significant number of nuclei can be measured in a few minutes (>3000 nuclei per min), which makes results statistically robust and representative of the whole population. A constraint for broad application of this type of flow cytometry analysis is complexity and cost of equipment. However, in recent years, modern, small and more affordable multiparameter flow cytometers have become readily available in many laboratories (Ochatt, 2008; Ochatt et al., 2011). Therefore, flow cytometry analysis of relative nuclear DNA content should be of broad application for assessing effect of stresses on elicitation of androgenesis of many species.
Although several decades have elapsed since the first reports on haploid plants from anthers/microspores, it is only recently that progress was made in understanding the molecular mechanisms underlying the switch from a gametophytic to a sporophytic path (Seguí-Simarro et al., 2006). While the identities of most of these genes remain unknown, in several cases they appear to be stress-related or are associated with zygotic embryogenesis. These stress-related genes may be involved with a general reprogramming of the cell or providing some type of protection from stress (Reynolds, 1997; Maraschin et al., 2005). Molecular approaches for the identification of embryogenesis genes have included for instance the use of gene expression profiling, and revealed the expression of several embryogenesis-related genes like the BABY BOOM ERF/AP2 transcription factor (Boutilier et al., 2002), LEC1 and LEC2 (Gaj et al., 2005; Braybrook et al., 2006; Fambrini et al., 2006; Alemanno et al., 2008), which took place as early as 48-72 h after initiating microspore culture. Already in their early study, Rech et al. (1988) had shown that DNA synthesis was enhanced by electroporation, and its use as an eliciting factor in our experiments here is likely to have encompassed an increased expression of some of the genes above.

Assessment of expression profiles of genes and transcription factors known to be involved in embryo development in the model species arabidopsis (Arabidopsis thaliana L.) and M. truncatula, is envisaged in order to get a better understanding of the genetic basis of doubled haploidy in pea. We are also presently analysing the effects of the various stress treatments on the level of G-C by using Chromomycine A3, which is GC-specific, and on the concomitant AT/ GC ratio of stressed anthers.
The results presented here form a solid basis for further efforts designed to enhance responses and to extend doubled haploid technology to other pea genotypes and to other legume species, generally regarded as recalcitrant to this approach.
3.5 References


Lülsdorf MM, Croser JS, Ochatt S (2011) Androgenesis and doubled haploid production in food legumes. CABI, Oxfordshire


Chapter 4

Antigibberellin-induced reduction of internode length favors in vitro flowering and seed-set in different pea genotypes

Disclaimer - The research presented in Chapter 4 has been published in Biologia Plantarum: Ribalta FM, Croser JS, Erskine W, Finneghan PM, Lülsdorf MM, Ochatt SJ (2014). Antigibberellin-induced reduction of internode length favors in vitro flowering and seed-set in different pea genotypes. Biol. Plant; Vol 58, Issue 1, pp. 39-43. All the glasshouse and laboratory work related to this publication was carried out solely by Federico Ribalta. The publication was written by Federico Ribalta and the co-authors were involved in the experimental planning phase, discussion of results, structure of the paper and editorial comments to finalise it.

4.1 Introduction

In vitro based modified single-seed-descent (SSD) systems have been proposed as one method to accelerate generation turnover across a number of species (Franklin et al., 2000; Ochatt et al., 2002; Asawaphan et al., 2005; Zhang, 2007; Ochatt and Sangwan, 2008). The in vitro SSD technique involves the shortening of generation time by culturing immature seeds after forced in vitro flowering. Current conventional SSD methodologies enable a maximum of three generations per year to be developed in pea (Pisum sativum L.) (Ochatt and Sangwan, 2010). Pea cultivars are usually released after 8-10 generations of self-pollination to achieve an appropriate level of homozygosity (Kasha and Maluszynski, 2003). Decreasing the length of the generation cycle will overcome this breeding bottleneck and accelerate genetic improvement. Doubled haploidy is the fastest known route to homozygosity (Chase, 1952), however, considerable further research is required before this technology will be available routinely within a pea breeding programme (Croser et al., 2006; Ochatt et al., 2009).

There have been three in vitro flowering protocols proposed for pea (Fujioka et al., 1999; Franklin et al., 2000; Ochatt et al., 2002); however, these protocols were
developed for a limited number of early-flowering cultivars. To enable the
development of a widely applicable protocol we studied the effect of *in vivo* versus *in vitro* application of the antagibberellin Flurprimidol to reduce internode length, of light
quality on *in vitro* flowering and seed-set, and of the optimization of culture
methodology through comparative *in vitro* culture of intact plants, plants with the
meristem removed or excised shoot tip explants. Based on our results we report
herewith an improved protocol that enables *in vitro* flowering across a range of pea
genotypes, including mid and late flowering types.

The antagibberellin Flurprimidol is a chemical used in horticulture to reduce
plant height and produce compact plants. It reduces internode elongation through the
inhibition of gibberellic acid (GA) biosynthesis (Rademacher, 2000). Flurprimidol has
been extensively used to control plant growth under glasshouse conditions in a
number of species (Hamid and Williams, 1997; Pobudkiewicz and Treder, 2006; Burton
et al., 2007), including pea (Ochatt et al., 2002). However, to our knowledge,
Flurprimidol has never been used in any *in vitro* flowering protocol by adding it to the
medium. We believe the addition of Flurprimidol *in vitro* will result in smaller plants
required for *in vitro* growth.

Environmental factors such as light quality (Reid et al., 1996; Cerdan and Chory,
2003; Ausín et al., 2005; Nelson et al., 2010), photoperiod length (Reid et al., 1996;
Ceunen and Geuns, 2013), growth temperature (Ausín et al., 2005; Nelson et al., 2010)
and stress (Wang et al., 2012; Zhou et al., 2012) play a key role in the regulation of the
transition to flowering in plants. Among these, in pea the effect of light quality is
unclear but exploitable. In general, light containing a mixture of red and far-red light is
most effective in causing flowering of long day plants (Reid and Murfet, 1977; Vince-
Prue, 1981; Weller et al., 1997; Ausín et al., 2005; Cummings et al., 2007). In 2001, Runkle and Heins reported the red: far-red ratio had no effect on *in vivo* flowering time of the long day pea cv. Utrillo. In contrast, Cummings et al. (2007) reported the high red: far-red ratio of light sources like cool white fluorescent and high-intensity discharge lamps delayed pea flowering *in vivo* and inhibited internode extension in long day genotypes. Thus, we reason the addition of far-red filtered light may help reduce the red: far-red ratio and thus stimulate *in vitro* flowering and seed development in pea.

To develop a protocol that could be widely adopted, it was necessary to optimize culture factors considered important to *in vitro* flowering and seed initiation and maturation. The factors we identified as critical were the gas exchange and humidity level through the use of different culture vessel types (Lentini et al., 1988; Fujioka et al., 1999; Asawaphan et al., 2005), explant sources (culture of intact plants, plants with the meristem removed or excised shoot tip explants) (Narasimhulu and Reddy, 1984; Franklin et al., 2000; Ochatt et al., 2000; 2002), culture medium composition (Franklin et al., 2000; Ochatt et al., 2000; 2002; Asawaphan et al., 2005), and the addition of plant growth regulators (Narasimhulu and Reddy, 1984; Chengalrayan et al., 1995; Franklin et al., 2000; Ochatt et al., 2002). We believe the optimization of these factors would lead to a robust pea *in vitro* flowering protocol.

The aim of this work is to understand the effect of physiological mechanisms operating *in vitro* on the efficient and reproducible induction of flowering and seed-set across a range of pea genotypes. The final goal is to contribute to the acceleration of generation cycles for faster breeding of novel genotypes.
4.2 Materials and methods

4.2.1 Plant material

A range of genetically diverse *Pisum sativum* L. genotypes with varying flowering times were selected for this research (Table 4.1).

**Table 4.1** Pea genotypes used in this study and their main characteristics.

<table>
<thead>
<tr>
<th>Name</th>
<th>Flowering type</th>
<th>Plant habit</th>
<th>Leaf type</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frisson</td>
<td>Early</td>
<td>Semi-dwarf</td>
<td>Conventional</td>
<td>French cv. (Ochatt et al., 2002 - control)</td>
</tr>
<tr>
<td>Excell</td>
<td>Early/Mid</td>
<td>Semi-dwarf</td>
<td>Semi-leafless</td>
<td>Australian cv.</td>
</tr>
<tr>
<td>Bundi</td>
<td>Early/Mid</td>
<td>Semi-dwarf</td>
<td>Semi-leafless</td>
<td>Australian cv.</td>
</tr>
<tr>
<td>Victor</td>
<td>Mid</td>
<td>Semi-dwarf</td>
<td>Conventional</td>
<td>French cv. (Ochatt et al., 2002)</td>
</tr>
<tr>
<td>Dunwa</td>
<td>Mid/late</td>
<td>Tall</td>
<td>Conventional</td>
<td>Australian cv.</td>
</tr>
<tr>
<td>Kaspia</td>
<td>Late</td>
<td>Semi-dwarf</td>
<td>Semi-leafless</td>
<td>Australian cv. (industry standard)</td>
</tr>
<tr>
<td>00P016-1</td>
<td>Very late</td>
<td>Tall</td>
<td>Conventional</td>
<td>Ethiopian germplasm accession: landrace with blackspot resistance</td>
</tr>
</tbody>
</table>

4.2.2 Flurprimidol experiments

4.2.2.1 *In vivo* experiments

Seeds of cv. Kaspia and line 00P016-1 were sown in a glasshouse at The University of Western Australia (Perth, Australia - lat: 31°58’49” S; long: 115°49’7” E) in 1 dm³ pots filled with UWA Plant Bio Mix potting mix (Richgro Garden Products, Perth, Australia). The temperature was controlled at 20/18°C day/night under ambient light conditions. In preliminary experiments it was observed that cv. Kaspia and line 00P016-1 did not flower under *in vitro* conditions; this is the reason why the *in vivo* approach was taken for these two genotypes.

Flurprimidol, 2-methyl-1-pyrimidine-5-yl-1-(4-trifluoromethoxy phenyl) propane-1-ol (Topflor, SePRO Corporation, Carmel, IN, USA), was used to reduce internode elongation. In order to identify the most effective Flurprimidol treatment, a 5% w/v solution was applied as a drench at various concentrations (0, 25, 50 and 75
Applications were repeated three times at 10-day intervals from the three-leaf stage (Ochatt et al., 2002).

### 4.2.2.2 In vitro experiments

Dry seeds were surface-sterilized by treatment for 5 min in 70% ethanol, followed by 10 min in sodium hypochlorite (21 g dm\(^{-3}\)). Seeds were rinsed three times with sterile deionised water and imbibed overnight. The coats of imbibed seeds were removed and 10 embryos with both cotyledons intact were cultured in a vessel containing 50 cm\(^3\) of B5 salts and vitamins (Gamborg et al., 1968) modified by the addition of 10 mM NH\(_4\)Cl (Ochatt et al., 2000).

After seven days, the shoot apical meristems (1 cm length and comprising two internodes) of 50% of the plants, randomly selected, were removed using a scalpel. Both, the plants with the meristem removed, which developed via axillary branching, and the excised shoot apical meristems were then individually cultured in vitro and compared to cultured intact plants. The three treatments, plants with the meristem removed, excised shoot tip explants and intact plants were cultured into either 150 x 25 mm borosilicate glass tubes covered with a polypropylene closure (PhytoTechnology Laboratories, Kansas, USA) and containing 20 cm\(^3\) of MS medium (Murashige and Skoog, 1962), or into 150 x 70 mm polycarbonate containers sealed with a screw cap (Sarstedt Australia Pty Ltd, Adelaide, Australia) with a 4 mm diameter hole covered in breathable membrane (Flora Laboratories, Melbourne, Australia) with 50 cm\(^3\) of MS medium. The pH of the medium was adjusted to 5.6 prior to autoclaving at 121°C for 20 min.

All cultures were incubated at 24°C with a light intensity of 145 µmol m\(^{-2}\) s\(^{-1}\) from cool-white-fluorescent tubes (LIFEMAX TL-D 30W/840, Philips Lighting, Bangkok,
Thailand), unless stated otherwise. Preliminary experiments included culture of explants at photoperiods of 12, 16, 20 and 24 h of light. However, there was no significant difference between treatments. Thus, a photoperiod of 20/4 h light/dark was chosen for all *in vitro* experiments in this study.

To evaluate the effect of Flurprimidol on *in vitro* growth and flowering, a range of concentrations (0, 5, 10, 15, 20, 30 and 35 cm$^3$ of 5% w/v Flurprimidol per dm$^3$ of medium) were added filter-sterilized to the MS medium immediately after autoclaving. *Also, experiments were undertaken to assess the effect of vernalisation on in vitro flowering in pea. However, no significant differences were observed between treatments (Appendix 3).*

### 4.2.3 Light quality treatments

To study the effect of light quality on *in vitro* flowering induction, three different light source types were compared: 1- Cool-white fluorescent light only (145 µmol m$^{-2}$ s$^{-1}$); 2- Cool-white fluorescent combined with red fluorescent light from F30W/GRO Gro-Lux tubes (Sylvania Lighting International, Erlangen, Germany) (120 µmol m$^{-2}$ s$^{-1}$); and 3- Cool-white fluorescent plus far-red filtered light (Blood Red 789, LEE filters, Andover, England) (130 µmol m$^{-2}$ s$^{-1}$).

In all experiments, flowering time and node number of the first flower were recorded. Also, the average internode length (average plant length/ average number of nodes) was measured at day 40. In addition, the efficiency of recovery of immature seeds and embryos was studied. This included the selection of the embryo developmental stage (number of days after flowering) for best *in vitro* germination and plant development. The immature pods at 16, 18, 20 and 22 d after flowering...
were opened aseptically and the embryos removed and directly transferred on to new
glass tubes containing 20 cm$^3$ of hormone-free MS medium.

The experimental design was completely randomized and all treatments were
repeated at least three times with a minimum 10 replicates per treatment per
genotype. Statistical analysis was performed by analysis of variance (Microsoft Office
Excel 2007 software).
4.3 Results

4.3.1 Effect of Flurprimidol on internode length and flowering time

Pea plant height was reduced under all conditions: the addition of the antigibberellin Flurprimidol produced smaller plants by reducing internode length in vivo (Appendix 4). For example, for line 00P016-1 we observed an average reduction in plant height from 77 ± 7.0 cm in the control treatment (0 cm$^3$ of Flurprimidol) to 13 ± 2.8 cm for the highest Flurprimidol concentration applied (75 cm$^3$). Flurprimidol also reduced internode length in vitro as shown in Fig. 4.1 for cv. Kaspa; although with lower concentrations of antigibberellin. Flurprimidol had no effect on flowering time and seed-set under either in vivo or in vitro conditions and no associated change in node number was observed.

Figure 4.1 Effect of Flurprimidol on internode length in vitro for different explant sources of cv. Kaspa at day 40 (results are expressed as mean ± SE; n = 30; P = 0.05).

4.3.2 Interaction Flurprimidol x environment

For both, in vivo and in vitro experiments, an important seasonal effect of Flurprimidol was detected. In the glasshouse under natural light conditions, internode length was reduced more in spring compared to autumn. For any given concentration
used, Flurprimidol was less effective in reducing plant size during the autumn-winter period (shorter days and lower light intensity) than in the spring-summer period (longer days and higher light intensity) (Appendix 4). Similarly, in controlled environment rooms under low light intensity (110-180 µmol m\(^{-2}\) s\(^{-1}\)) Flurprimidol was less effective in reducing plant size \textit{in vitro}, therefore a higher concentration was required than under a higher light intensity (550-900 µmol m\(^{-2}\) s\(^{-1}\)) to produce similar effects. Thus, under low light intensity the addition of Flurprimidol \textit{in vitro} at a concentration of 30 cm\(^3\) dm\(^{-3}\) reduced the average plant size at day 40 of cv. Kaspa by 20.9%, while under higher light intensity a concentration of 15 cm\(^3\) dm\(^{-3}\) was sufficient to produce similar plant size reduction.

\textbf{4.3.3 Interaction Flurprimidol x genotype}

The effectiveness of Flurprimidol on reducing plant height varied across genotypes. Flurprimidol had a greater effect on tall genotypes compared to semi-dwarf genotypes. For example, for the tall landrace 00P016-1 a 25 cm\(^3\) application of the Flurprimidol solution under glasshouse conditions reduced plant height by 73.6% compared to the control treatment (0 cm\(^3\) of Flurprimidol). Conversely, the same Flurprimidol treatment reduced plant height by just 37.9% for the semi-dwarf cv. Kaspa.

\textbf{4.3.4 Effect of Flurprimidol on different culture explant sources}

Flurprimidol significantly reduced \textit{in vitro} internode length on the three explant sources studied: intact plants; plants with the meristem removed and excised shoot tip explants (Fig. 4.1). Plants with the meristem removed developed through the proliferation of a cotyledonary node axillary meristem. No significant difference was observed on \textit{in vitro} time to flowering when comparing intact plants and plants with
the meristem removed. Thus, for plants of cv. Excell grown in tubes, the average flowering time was $32 \pm 2.7$ d for intact plants and $32 \pm 5.9$ d for plants with the meristem removed. In contrast, excised shoot tip explants cultured \textit{in vitro} always took longer to flower, e.g. the average flowering time of excised shoot tip explants of cv. Excell grown in tubes was $44 \pm 5.5$ d. It was observed that only excised shoot tip explants that produced roots were able to form flowers.

### 4.3.5 Light quality effect

No significant effect of light quality \textit{in vitro} was observed on: a- days to flowering, b- percentage of flowering plants, and c- percentage of podding plants (Appendix 4). White fluorescent light was thus adopted in the subsequent experiments. For all genotypes studied, light quality treatments, and Flurprimidol treatments, the node of first flower production was not affected. For each treatment the mean node of first flower production for intact plants of cv. Excell was $12 \pm 0.4$.

### 4.3.6 Culture system

In general, there was a higher percentage of seed-set in plants grown in tubes compared to plants grown in containers (Fig. 4.2), despite more flowers being produced in containers (Fig. 4.3). The largest difference between treatments was observed for conventional leafed cvs. Frisson and Victor, with a consistently higher percentage of seed setting plants in tubes compared to containers. However, a lower difference between treatments was observed for the semi-leafless cvs. Excell and Bundi. Interestingly, for intact plants of cvs. Excell and Bundi, containers favoured marginally better seed-set than tubes. The type of culture vessel used had no effect on plant architecture irrespectively of the genotype studied.
Figure 4.2 Percentage of plants that set seeds under white fluorescent light (results are expressed as mean ± SE; n = 30; T: tubes; C: containers).

Figure 4.3 Number of flowers per plant produced in vitro from different explant sources for A - cv. Frisson and B - cv. Excell (results are expressed as mean ± SE; n = 30; P = 0.05; MR: meristem removed; STE: shoot tip explants; IP: intact plants).

Flurprimidol concentration: Intact plants and plants with the meristem (30 cm⁻³ dm⁻³), shoot tip explants: (10 cm⁻³ dm⁻³).

In order to shorten the length of the reproductive cycle, immature embryos were removed and cultured in vitro. Best germination rates were obtained by culturing immature seeds between 18-20 d after flowering. The length of the...
generation cycle in vitro was approximately 50 d for most of the genotypes studied (Victor: 48 ± 1.8 d; Bundi: 49 ± 3.6 d; Excell: 50 ± 5.9 d; and Frisson: 54 ± 3.4 d). On the other hand, for the very late flowering landrace 00P016-1, the average generation length in vitro was over 90 d with a very low flowering and seed setting rate (under 10%).

4.3.7 Comparison of growth environments

Significant differences among the studied genotypes were observed in flowering time and in the estimated average generation cycle length when comparing plants grown under different growth environments (Fig. 4.4). With the proposed in vitro flowering protocol, up to seven generations per year can be obtained for the early/mid flowering cvs. Bundi and Excell; and over five generations for the mid/late flowering cv. Dunwa.

![Figure 4.4](image-url) Comparison between growth environments for estimates of average generation cycle length for different pea cultivars. Numbers within bars indicate average flowering time in days (data are means from three repetitions with at least 10 individuals). Flowering time in the field source: Department of Agriculture and Food of Western Australia, Australia.
4.4 Discussion

An optimized *in vitro* flowering protocol is presented here using Flurprimidol to control *in vitro* plant size, culturing plants with the meristem removed and excised shoot tip explants into glass tubes under white fluorescent light. *In vitro* flowering and seed-set was achieved across a genetically diverse range of pea genotypes (Appendix 5) with an average generation cycle length of approximately 50 d for the early to mid flowering cultivars. We were able to accept our hypothesis that the antigibberellin Flurprimidol permits *in vitro* flowering across a range of pea genotypes by reducing internode length. However, the addition of red or far-red light sources did not reduce the flowering time *in vitro* in the genotypes studied.

In order to shorten the generation cycle of pea, it is essential to control plant growth to obtain plants with reduced vegetative development (Ochatt et al., 2002). In horticulture Flurprimidol has been used *in vivo* to produce compact plants. This chemical acts by blocking cytochrome P450-dependent monooxygenases, catalyzing the oxidation of ent-kaurene into ent-kaurenoic acid, thereby inhibiting GA biosynthesis (Rademacher, 2000). In our experiments, Flurprimidol reduced pea plant size *in vivo* and *in vitro*. In terms of logistics, Flurprimidol permits growth *in vitro* by producing smaller plants, which is particularly important for tall genotypes.

Internode length in pea is determined at least by five major loci: *Le*, *La*, *Cry*, *Na*, *Lm* (Reid et al., 1983). GA$_1$ is the native GA controlling internode elongation in pea (Ross et al., 1989). Slender phenotypes (*la*, *cry*) have long and thin internodes and are not dependent on endogenous GAs. Dwarf plants (*La* and/or *Cry*) acquire a similar phenotype to slender types when treated with high concentrations of GA$_3$ (Potts et al., 1985). A major gene (*Le/le*) for internode length is mainly responsible for tall climbing...
(Le/-) versus dwarf bush (le/le) habit (Potts et al., 1982). Application of GA\(_1\) can mask the Le/le gene difference. Le plants respond equally to GA\(_{20}\) and GA\(_1\), while le plants respond only weakly to GA\(_{20}\), the major biologically active gibberellin found in dwarf peas. These results suggest that the Le gene controls the production of a 3β-hydroxylase capable of converting GA\(_{20}\) to GA\(_1\) (Ingram et al., 1983).

As evoked above, gibberellins are a large group of compounds which share a common gibbane ring structure, and which all have at least some physiological activity (Cleland, 1999). They include both precursors and catabolites and thus, even if GA\(_1\) is the main active GA in stem elongation (Ingram et al., 1986), other GAs are as active or more active in processes including, among others, tendril and pod growth in pea (Smith et al., 1992). Thus, while the biosynthesis of GA\(_{20}\) takes place in unfolded leaves and tendrils, its conversion to GA\(_1\) occurs in the upper stem (Smith, 1992). The paramount role of GAs on leaf expansion has been proven in cereals (Nelissen et al., 2012) and is also known in pea (Hedden, 1999). In this context, the differential responses observed between the different tissues tested and also between the conventional (Frisson and Victor) and semi-leafless (Bundi and Excell) cultivars might be attributed to a differential endogenous content of active GAs within the tissues in conjunction with the antigibberellin effect of the exogenously applied Flurprimidol.

For long day plants, a red to far-red ratio close to the natural level of around 1 is the most effective for flower induction (Vince-Prue, 1981). Most growth chamber light sources have high red: far-red ratios (greater than 2) which can delay flowering in photoperiodic sensitive species and inhibit internode extension (Whitman et al., 1998; Runkle and Heins, 2001; Cummings et al., 2007). In our experiments, the addition of red or far-red light sources did not reduce the flowering time in vitro in early, mid or
late flowering pea genotypes. The addition of far-red filtered light probably did not
correct the red: far-red ratio sufficiently as to mimic natural light, as was discussed by
Runkle and Heins (2001) and Cummings et al., (2007). Also, the light intensity applied
may not have been high enough to induce fast in vitro flowering in the genotypes
studied (Fujioka et al., 1999). It was then concluded that white fluorescent light was
the best of the light sources tested in the in vitro flowering protocol presented here.

Growth rates, and many of the physiological characteristics of plants developed
in vitro, are influenced by the physical and chemical environment of the culture
vessels (Walker et al., 1988; Jackson et al., 1991; Kozai et al., 1992; Majada et al.,
1997). The type of culture vessel and its closure (use of gas-permeable membranes or
lids) influences or alters the in vitro environment by modifying the gas composition.
When comparing different culture vessels, we observed a higher percentage of seed-
setting plants when culturing in tubes covered with polypropylene caps compared to
containers sealed with screw caps covered in a breathable membrane. This is likely to
be associated with better air exchange (reduced levels of ethylene and CO₂
concentration) of glass tubes compared to polycarbonate containers, as reported by
Jackson et al. (1991) and Lentini et al. (1988). Interestingly, we observed that plants
grown in tubes produced a lower number of flowers compared to plants grown in
containers, and that this was true irrespectively of their flowering or leaf type, as the
early, leafy type cv. Frisson gave the same responses as the early/ mid, semi-leafless
type cv. Excell (Fig. 4.3). This may be related to the smaller amount of culture medium
used in tubes compared to containers, which may affect the partition of nutrients in
the plant, thus affecting the production of flowers, as observed previously with other
species (Figueira and Janick, 1994; George et al., 2009). Therefore, tubes were
considered the best culture vessel in the \textit{in vitro} flowering protocol proposed in this work, as the reduced number of flowers per plant produced was also associated with a lesser early pod abortion, maybe due to a reduced competition between flowers.

Efficient breeding methods are needed to advance hybrid populations and to facilitate selection of lines with desirable combinations of characters (Haddad and Muehlbauer, 1981). The SSD method consists of taking a single seed from each $F_2$ plant and advancing each seed to the next generation until a desired level of homozygosity is achieved ($F_6$ to $F_8$), thus saving space and time (Goulden, 1939). However, the extent of plant loss from generation to generation affects the genetic makeup of the SSD populations (Martin et al., 1978). We have calculated that with an attrition rate per generation of 10\% of the plant population, after four generations of SSD (from $F_2$ to $F_6$) we would end up with only 34\% of the initial population. This indicates the importance for the breeder of the robustness and reliability of any tissue culture system used. In the present study, removing the meristem and culturing it separately permits the production of a second cloned plant, which provides a back-up plant in case of loss. This is particularly important when working with rare and valuable genotypes in a population such as the production of recombinant inbred lines (Soller and Beckmann, 1990).

The identification of a system to accelerate generation turnover by shortening each cycle is crucial in breeding programmes. In this publication we present a simple and reliable system to reduce generation time \textit{in vitro} across a range of pea genotypes. In this protocol Flurprimidol is used to control plant size \textit{in vitro}, and plants with the meristem removed and shoot tip explants are cultured into glass tubes under cool-white fluorescent light. More than five generations per year can be obtained with
mid to late flowering genotypes using this protocol and over six generations per year for early to mid flowering genotypes. However, some late/very late flowering genotypes like cv. Kaspa and the landrace accession 00P016-1 remain recalcitrant to this technology. The presented data provides the possibility to answer more basic questions related to *in vitro* plant growth and development via the exploration of the endogenous changes in phytohormone levels in genetically diverse peas after Flurprimidol application.
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Chapter 5
General discussion and future directions

In plant breeding, the development of varieties with resistance to abiotic and biotic stress is a very costly and time consuming process. In order to accelerate the genetic progress it is essential to provide plant breeders with the broadest variety of biotechnological tools. Two in vitro technologies have been proposed for the rapid achievement of homozygosity and thus acceleration of the breeding process in many economically important crops: a) Doubled haploidy, and b) an in vitro-based modified single-seed-descent (SSD) system. In pea (Pisum sativum L.), neither of these technologies is routinely used in a breeding program. The aim of this study was to accelerate the breeding process in pea by developing in vitro techniques to rapidly achieve a high level of homozygosity and to better understand the fundamental mechanisms involved in these processes.

5.1 Doubled haploidy

Despite the significant effort undertaken in this area, to date there is no universal doubled haploid (DH) protocol effective in every species and success remains highly genotype-dependent. Leguminous species, including pea, have been described as recalcitrant to this technology (Croser et al., 2006; Dita et al., 2006; Lülsdorf et al., 2011). Since the first DH studies in pea undertaken by Gupta et al. (1972) many researchers have worked toward a robust DH protocol with very limited success. Recently, Ochatt et al. (2009) obtained a few haploid pea plantlets confirmed by flow cytometry. However, these plantlets were weak and did not survive glasshouse transfer. It is notable that after more than forty years of international research effort, no robust DH protocol has been developed in pea or any other legume species and
little is known about the fundamental mechanisms involved in androgenesis elicitation.

In the first part of this research thesis (Chapter 2), a number of key factors known to be involved in androgenesis elicitation were identified and optimised in order to develop a robust and efficient anther culture protocol for pea. In this study, the combined application of multiple stress treatments, including the novel stress agent sonication, applied at the appropriate time (uni-nucleate microspore developmental stage) and the optimisation of key culture factors (i.e. light intensity and use of plant growth regulators) led to the development of an efficient protocol for the routine induction of androgenesis and advanced-stage embryo production from extracted anthers of genetically diverse pea genotypes.

The requirement of hormones for the induction of haploid divisions in pea is still unclear. Ochatt et al. (2009) indicated that the hormonal composition of the basal medium had little influence on either initial or further proliferation of isolated microspore culture for pea. In contrast, Bobkov (2010) obtained callus production from extracted pea anthers by culturing in medium supplemented with 2,4-D. The flow cytometry analysis presented in Chapter 3 showed that the application of multiple stress treatments, with no exogenous hormones, permitted the induction of androgenesis from cultured anthers of pea. From these results it can be inferred that stress is indeed the trigger diverting microspores from their normal development in pea and that hormones are not required for the induction of androgenesis from cultured anthers. In the present research, the addition of hormones to the culture medium promoted callus initiation after stress induction of microspores.
It was clear from this study that it was the combined application of multiple stress treatments, including a cold pre-treatment, electroporation, centrifugation, osmotic shock and the novel stress sonication, which triggered androgenesis elicitation from cultured anthers of pea. The benefit of pyramiding multiple stress treatments on the induction of haploid divisions has also been reported in isolated microspore culture for pea (Ochatt et al., 2009) and anther culture for chickpea (*Cicer arietinum* L.) (Grewal et al., 2009). Little is known about the exact mechanisms underlying elicitation of androgenesis by stress treatments. While many attempts have been made to explain the possible effects of some of these stress factors (reviewed by Shariatpanahi et al., 2006) it has been difficult to evaluate the effect of these stress treatments on microspore DNA synthesis. Thus, in Chapter 3, a flow cytometry study was undertaken to further understand the effect of individual and combined stress treatments on androgenesis elicitation. Analysis of the flow cytometry results revealed clear differences in the relative nuclear DNA content of microspores within anthers after stress treatments. Based on these results, stress treatments could, for the first time, be differentiated between those that elicit androgenesis (cold, electroporation and osmolarity) and those that enhance DNA synthesis following elicitation (centrifugation and sonication). The publication of this finding has established flow cytometry as a method to quickly assess the effect of individual and combined stress treatments, based on the relative nuclear DNA content (Ribalta et al., 2012). This technique will be particularly useful in assisting researchers to rapidly identify effective stress treatments during DH protocol development, not only for legumes but also for other plant species.
On the basis of the research presented in Chapters 2 and 3, the following protocol for the elicitation of androgenesis and haploid embryo production from extracted anthers of pea is proposed:

- Harvest buds 6-7 mm in length (when microspores are at the uni-nucleate stage). Microscopic analysis of stage of microsporogenesis is recommended when working with new genotypes.
- Apply a cold pre-treatment to buds in the dark at 4°C for a minimum of 48 h (but no longer than 14 d).
- Sterilise buds for 1 min in 70% v/v ethanol, followed by 10 min 1% v/v in commercial sodium hypochlorite. Rinse buds three times in sterile water.
- Remove anthers from buds, ensuring complete removal of filament and apply the following stress treatments prior to culture: electroporation (three successive pulses at 10 s intervals of 1000-1500 V/cm with a capacitance of 100 µF and a resistance of 50 Ω), centrifugation (170 g for 10 min at 4°C, brakes off) and sonication (38 kHz for 30 s at room temperature).
- Place 10 anthers per treatment in a 10 x 35 mm Petri dish containing 2 ml of liquid medium containing 17% (w/v) sucrose and incubate at 24°C in the dark. After 1 week replace culture medium with fresh medium containing 10% (w/v) sucrose.
- After 4 weeks of culture in the dark transferred calli to the Loiseau et al. (1995) media sequence for embryo germination and maturation.

5.1.1 Future directions

In this research, key factors involved in haploid embryogenesis were identified and optimised in pea. However, additional research is still required before this technology will be routinely available within a pea breeding program. Further
improvement in the development of DH protocols and the expansion to recalcitrant species can be expected with a more thorough understanding of the fundamental mechanisms involved in androgenesis. Doubled haploid research still lacks a legume model species that would enable us to gain a better fundamental understanding of the haploid embryogenesis process in legumes and lead to improved protocols.

Despite these limitations, in recent times regeneration via overexpression of genes, such as \textit{WUSCHEL (WUS)} or \textit{LEAFY COTYLEDON (LEC)}, in \textit{Arabidopsis thaliana} (L.) Heynh. has started to provide a basis for understanding the genes involved in somatic embryogenesis (Rose and Nolan, 2006). The \textit{LEC} genes \textit{LEC1}, \textit{LEC2} as well as \textit{FUSCA3}, have major effects on embryo development (Harada, 2001). \textit{LEC} transcription factors establish environments that promote cellular processes characteristic of the maturation phase and the initiation of somatic embryogenesis formation (Braybrook and Harada, 2008). Ectopic expression of \textit{LEC1} and \textit{LEC2} induces somatic embryogenesis in the absence of exogenous auxin or stress treatments (Lotan et al., 1998; Stone et al., 2001), while the ectopic expression of \textit{FUSCA3} results in elevated abscisic acid (ABA) levels (Braybrook and Harada, 2008). Interestingly, the presence or absence of these two groups of growth regulators was recently studied to unravel the progression of mitosis and the onset of endoreduplication (indicative of the transition from the cell division to the storage product accumulation phase) in immature seeds and excised embryos of \textit{Medicago truncatula} Gaernt. (Ochatt, 2011; Atif et al., 2013). These studies showed that the establishment of such transition and the onset of storage product accumulation were required for early embryo development and subsequent germination. The ectopic expression of the transcription factors \textit{WUS} and \textit{BABY BOOM (BBM)} have also been implicated in the induction of somatic embryos in
Arabidopsis (Rose and Nolan, 2006). Future research should concentrate on the identification and isolation of selected candidate genes involved in somatic embryogenesis in model species like A. thaliana and M. truncatula during the acquisition of androgenesis competence by microspores. These studies will provide valuable information on how the androgenesis process is controlled in different species and under different culture conditions.

There is now a growing body of evidence that it is the application of multiple stress agents that will overcome recalcitrance in legumes to androgenic induction. As discussed in Chapter 2, this is likely to be due to the increasing hormone levels in stressed anthers. Androgenesis has been shown to be mediated through ABA levels in a number of species (Imamura and Harada, 1980; Van Berger et al., 1999; Wang et al., 2000; Żur et al., 2008); however, other hormones, such as gibberellins, auxins and cytokinins, have also been associated (Brugière et al., 2003; Maraschin et al., 2005).

Recently, Lülsdorf et al. (2012) showed a possible link between androgenesis induction and auxin levels, particularly IAA-asparagine (IAA-Asp), in stress-treated anthers of pea and chickpea. More research is now required to further elucidate the effect of endogenous hormone levels on androgenesis elicitation. The improved knowledge on the role of endogenous hormones on the induction of haploid divisions, together with a more thorough understanding of the genetic basis of androgenesis will have a significant impact in the improvement of available DH protocols.

Success in the development of DH technology has been proportional to the number of laboratories involved and the availability of research funding. In the most frequently studied species, progress in DH protocol development has been the result of the sustained, combined effort across research institutes worldwide. In comparison,
a relatively small number of research groups have undertaken DH research in
legumes. Further progress in DH protocol development in legume species will require
collaboration among scientists from different research institutes. It is reasonable to
expect that given enough resources robust DH protocols will be developed for non-
amenable species such as the large-seeded legumes.

Although significant progress has been achieved over the past few years in the
development of DH protocols for the legumes pea, chickpea and *Lathyrus*, a more
thorough understanding of the fundamental mechanisms involved in microspore
embryogenesis is still required. The advent of modern biotechnological tools for high-
throughput functional genomics will greatly facilitate the identification and isolation
of genes involved in androgenesis elicitation. A more comprehensive understanding of
the genetic basis of androgenesis together with improved *in vitro* culture techniques
will facilitate the development of more efficient DH protocols across a range of
species, particularly for intractable species such as the large-seeded legumes.

### 5.2 In vitro based Single-Seed-Descent

*In vitro* based modified single-seed-descent (SSD) systems have been proposed
as an effective method to accelerate generation turnover across a number of species
(Franklin et al., 2000; Ochatt et al., 2002; Asawaphan et al., 2005; Zhang, 2007; Ochatt
and Sangwan, 2008). In pea, only three *in vitro* flowering protocols have been
published (Fujioka et al., 1999; Franklin et al., 2000; Ochatt et al., 2002) and these
have been developed for a limited number of early-flowering cultivars. While a
considerable number of publications exist in the area of *in vitro* SSD and proof of
concept has been established across a number of species, there is no evidence of this
technology being integrated into a breeding program. This is likely due to genotype-specificity, but this is not recorded in the existing publications.

As stated earlier, one of the aims of this research was to develop a robust protocol that would enable the integration of this technology on a broader scale within a pea improvement program. In Chapter 4, an optimised *in vitro* based SSD system was developed which enabled rapid *in vitro* flowering and seed-set across a range of pea genotypes including, for the first time, mid to late flowering types (Ribalta et al., 2014). In the proposed protocol, the antigibberellin Flurprimidol was used to control *in vitro* plant size, and plants with the meristem removed or excised shoot-tip explants were cultured into glass tubes under white fluorescent light. With this strategy more than five generations per year were obtained with mid to late flowering genotypes and over six generations per year for early to mid flowering genotypes.

The use of the antigibberellin Flurprimidol to control plant growth under glasshouse conditions has been previously reported in a number of species (Hamid and Williams, 1997; Pobudkiewicz and Treder, 2006; Burton et al., 2007), including pea (Ochatt et al., 2002). In Chapter 4, the use of Flurprimidol in an *in vitro* flowering protocol is reported for the first time (Ribalta et al., 2014). In the present study, the addition of Flurprimidol to the culture medium resulted in the smaller plants required for *in vitro* growth, thus permitting *in vitro* flowering across a range of pea cultivars. This is particularly important for *in vitro* culture of tall genotypes. Interestingly, differential responses were observed between the different tissues tested (intact plants, plants with the meristem removed and shoot tip explants) and also between the conventional (Frisson and Victor) and semi-leafless (Bundi and Excell) cultivars.
These differences were attributed to the variation in endogenous content of active GAs within the tissues in combination with the antigibberellin effect of the exogenously applied Flurprimidol.

The effect of environmental factors such as light quality, photoperiod, and growth temperature, on the transition to flowering has been extensively explored (Reid et al., 1996; Cerdan and Chory, 2003; Ausín et al., 2005; Nelson et al., 2010). While the effect of most of these factors is fairly well understood, the effect of light quality on flower initiation in pea is still unclear (Runkle and Heins, 2001; Cummings et al., 2007). In the present study, the hypothesis that the addition of far-red filtered light would help reduce the red: far-red ratio and thus stimulate in vitro flowering and seed development in pea was rejected. The addition of far-red filtered light probably did not correct the red: far-red ratio sufficiently to mimic natural light, as discussed by Runkle and Heins (2001) and Cummings et al. (2007). It was then concluded that white fluorescent light was the best of the light sources tested in the proposed in vitro flowering protocol.

Other key findings from the present study were the important effect of the type of culture vessel (glass tubes vs. plastic containers) and its closure (use of gas-permeable membrane or lids) on in vitro plant growth and development, likely associated with better gas exchange in glass tubes compared to containers as reported by Jackson et al. (1991) and Lentini et al. (1988). In this study, a higher percentage of seed-setting plants were observed when culturing in tubes covered with polypropylene lids compared to containers sealed with screw caps covered in a breathable membrane. Interestingly, a lower number of flowers were observed in plants grown in tubes compared to those grown in containers, probably due to the
reduced amount of culture medium in tubes. This decrease in flower number was linked to lower early pod abortion due to reduced competition between flowers. From these results, glass tubes were considered the best culture vessel in the proposed *in vitro* flowering protocol irrespective of the pea genotype studied.

The reliability of any tissue culture system is one of main requirements for the efficient application of this technology in a plant breeding program. A key aspect of the *in vitro* flowering protocol proposed herein is the removal and separate culture of the meristem of each plant, permitting the production of a second cloned plant and the provision of a ‘back-up’ plant in case of loss. This ‘back-up system’ has not been proposed previously and will be of particular importance when working with rare and valuable genotypes and when using the protocols to rapidly produce a population of recombinant inbred lines.

**5.2.1 Future directions**

Some late and very-late flowering genotypes of pea remain unresponsive to the *in vitro* flowering technology and further research is required to expand this protocol to these genotypes. A combined *in vivo/ in vitro* strategy has been used in legumes like pea and bambara groundnut (*Vigna subterranea* L.) (Ochatt et al., 2002), as well as in *A. thaliana* (Ochatt and Sangwan, 2008) to reduce the length of each generation cycle. This approach consists of alternating a first step *in vitro* for germination and a second step *in vivo* for full development. In the early flowering pea cv. Frisson more than five generations per year have been obtained with this approach (Ochatt et al., 2002). For late and very late flowering pea genotypes, the development of a combined *in vivo/ in vitro* strategy may offer a valuable alternative for the shortening of generation cycle.
In vitro selection has been used as a tool for the screening of a number of different abiotic stresses across a range of species (Samantaray et al., 1999; Zair et al., 2003; Flowers, 2004). However, this technology has not been adopted on a broad scale to legumes due to their general difficulty to culture in vitro (Dita et al., 2006). The in vitro flowering technology developed in this study offers the capacity to add an in vitro screen for key abiotic stresses into the culture protocol. For example, in pea boron toxicity is an important disorder that can limit plant growth (Bagheri et al., 1992). With the in vitro flowering technology a screening step for boron tolerance could be incorporated into the protocol at the immature embryo stage leading to an efficient integration of tolerant/ resistant germplasm into the breeding program of pea. The development of novel in vitro breeding tools for accelerating legume crop improvement will permit a timely response to emerging biotic and abiotic threats.

Another potential application for the in vitro flowering strategy is the development of genetically modified organisms (GMOs) for the fixation of transgenes in a riskless in vitro environment. Further, it can be applied in marker-assisted selection in breeding programs. Additionally, the in vitro flowering study presented herein provides the possibility to answer fundamental questions related to in vitro plant growth and development via the exploration of the endogenous changes in phytohormone levels under different growth conditions. Thus, the in vitro flowering technology is a fascinating area of research and has potential not only for the rapid fixation of new traits, but also for fundamental studies on flower regulation, mineral nutrition, hormonal manipulation, plant physiology, plant transformation and functional genomics.
5.3 Final conclusions

The potential of doubled haploidy in plant breeding is clearly evident. Doubled haploid systems are broadly applied in plant breeding, genetic mapping, mutation and transformation studies and in many areas of fundamental biology in a number of species, particularly *Brassica* spp. and cereals. However, legumes are still considered unresponsive to this technology. The broad application of the DH technology in legumes species will require the development of robust, genotype-independent protocols. In this research, key factors involved in androgenesis elicitation were identified and optimised in order to develop a reliable pea anther culture protocol. The application of multiple stress treatments, including the novel stress sonication and the optimisation of culture conditions, led to the routine induction of haploid division and the production of advanced-stage embryos from cultured anthers of pea. A flow cytometry study permitted, for the first time, the assessment and differentiation of the effect of stress on the elicitation of androgenesis based on relative nuclear DNA content of microspores. The results presented in this research form a solid platform for further efforts designed to enhance androgenic response in pea and to extend this technology to other legumes.

In fulfilment of the second objective of this research, an optimised *in vitro* based SSD system was developed which enabled rapid *in vitro* flowering and seed-set across a range of pea genotypes including, for the first time, mid to late-flowering types. This research resulted in a strategy to obtain more than five generations per year with mid to late-flowering genotypes and over six generations per year for early to mid-flowering genotypes. In the absence of a robust DH protocol, the *in vitro* based SSD system reported herein will offer a valuable alternative method for the rapid
achievement of homozygosity by shortening each generation cycle. In addition, this technology has excellent potential as a breeding tool for the screening of key abiotic stresses in the development of tolerant/ resistant germplasm; as well as in transformation studies and fundamental studies related to \textit{in vitro} plant growth and development. The studies undertaken within this thesis have contributed valuable knowledge to the doubled haploidy and \textit{in vitro} flowering research areas and are expected to have a significant impact on the acceleration of the breeding process in pea.
5.4 References


Appendix 1

**Electroporation buffers**

<table>
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<th>Components</th>
<th>Ochatt et al., 2009</th>
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<tr>
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<tr>
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</table>

**Culture media composition**

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<tr>
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**Hormones:** 2,4-D (1 mg/L); Picloram (0.25 mg/L); BAP (0.1 mg/L)
Appendix 2 – Doubled haploid research

Bud sterilisation and microspore isolation protocol:

Flower buds were placed in a 100 ml beaker (100 buds per treatment) and surface sterilised for 1 min in 70% v/v ethanol, followed by 10 min in calcium hypochlorite (70 gL⁻¹). Buds were rinsed three times with sterile water. Buds were then transferred to a sterile mortar and macerated with a pestle in 2ml of Rech et al. (1987) electroporation buffer. The electroporation buffer was used for the maceration of buds as the electroporation treatment followed the microspore isolation procedure. Three successive steps of suspension and centrifugation (100 g, 10°C, 5 min each) were used to eliminate debris remaining after serial sieving through 80, 60 and 40 µm filters (Ochatt et al., 2009).

Electroporation protocol

Isolated microspores were submitted to the electroporation treatment immediately after the isolation procedure in order to preserve their viability and to avoid sedimentation in the bottom of the cuvette

Electroporation cuvettes: The size of the cuvettes is determined by the gap width between electrodes. Cuvettes with electrodes 2 mm apart (model No. 620) were used in these experiments.

Role of electroporation buffer: For the application of electroporation treatments, two primary parameters need to be optimised, field strength and pulse length. Field strength is measured as voltage delivered across an electrode gap and relates to the potential difference experienced by the cell membrane in the electric field. When the induced potential reaches a critical value, a reversible breakdown of the cell membrane occurs resulting in the creation of temporary pores that allow nutrients from the culture medium to get into the cells (Zimmermann et al., 1976; Kinosita and Tsong, 1977), thus improving regeneration competence from microspores (Delaitre et al., 2001; Ochatt et al., 2009) and protoplasts (Rech et al., 1987; Ochatt et al., 1988) of various species. The electroporation buffer acts as a membrane protector preventing the creation of irreversible pores that led to cellular death (Ochatt et al., 1988; Delaitre et al., 2001; Quecini et al., 2002). Also, the conductivity of the electroporation buffer affects the value of the pulse length. In general, the more conductive the media is, the lower the pulse length which should be used.

Flow cytometry analysis

Sample preparation: Nuclei were mechanically isolated by gently chopping tissues (30 anthers per treatment and 20 mg of young leaf tissue for the control) for 30 seconds with a razor blade in 2 mL of single-step isolation + stain buffer (Partec®) containing 4,6 diamidino-2-phenylindole (DAPI). The suspension was then filtered through a 50 µm nylon mesh and immediately analysed using the flow cytometer.
Appendix 3 – \textit{In vitro} flowering experiments

\textbf{In vivo Flurprimidol experiments:} In order to identify the most effective Flurprimidol treatment, a 5\% w/v solution was applied as a drench on the soil at various concentrations (0, 25, 50 and 75 cm$^3$).

\textbf{In vitro Flurprimidol experiments:} Dry seeds (30 per treatment) were surface-sterilised by treatment for 5 min in 70\% ethanol, followed by 10 min in sodium hypochlorite (21 g dm$^{-3}$). Seeds were rinsed three times for one minute with sterile deionised water and imbibed overnight. The coats of imbibed seeds were removed and 10 embryos with both cotyledons intact were cultured in a vessel containing 50 cm$^3$ of B5 salts and vitamins (Gamborg et al., 1968) modified by the addition of 10 mM NH$_4$Cl (Ochatt et al., 2000) and 40 g dm$^{-3}$ sucrose. The pH of the medium was 5.6.

\textbf{Vernalisation experiments:} A series of experiments was undertaken in order to assess the effect of a vernalisation treatment on \textit{in vitro} flowering in the early/ mid flowering pea cv. Excell and in the late flowering cv. Kaspa. Seeds were sterilised and imbibed overnight as reported in section 4.2. The coats of imbibed seeds were removed and 10 embryos with both cotyledons intact were placed in a vessel containing 50 cm$^3$ of modified B5 medium and submitted to a cold treatment (4°C) for 0, 7, 14 and 21 d in the dark. After this period, the seeds were transferred to a culture room at 24°C with a light intensity of 145 \mu mol m$^{-2}$ s$^{-1}$ from cool-white-fluorescent tubes (LIFEMAX TL-D 30W/840, Philips Lighting, Thailand) and a photoperiod of 20/4 h light/dark. In the present experiments, the vernalisation did not have a significant effect on flowering time, percentage of flowering plants and number of nodes to first flower in the studied pea cultivars.
Appendix 4

Effect of Flurprimidol concentration at day 40 in glasshouse-grown pea landrace 00P016-1: A - Seasonal effect on internode length (cm) (results are expressed as mean ± SD; n = 30). B - Effect on plant size (all bars = 5 cm).

Effect of light quality and culture vessel type on time to flowering *in vitro* in cv. Frisson (Cont.: containers; W: white light; W+R: white + red light; W+FR: white + far red light).
Appendix 5

With the proposed protocol, in vitro flowering and seed-set was achieved across a range of pea genotypes, including cvs. Frisson and Bundi (early flowering), cv. Excell (early/ mid flowering), cv. Kaspa (late flowering) and the landrace 00P016-1 (very late flowering).