ANTHER CULTURE OF *SOLANUM* GENOTYPES

by

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ABSTRACT

Being the third most cultivated crop in South Africa, potatoes are of great economic importance. As potatoes originated from cooler areas in the world, they do not easily adapt to South African conditions. The main objective of potato breeding is, therefore, to extend the crop's limited genetic base. Progress in crop improvement is slow due to dominance, segregation and other factors caused by the tetraploid character of cultivated potatoes. A new breeding program for rapid progress has been initiated at the Vegetable and Ornamental Plant Institute, Roodeplaat, South Africa, which comprises the combination of conventional and unconventional breeding techniques. The program is based on the reduction of the ploidy level from the tetraploid to the dihaploid level to facilitate crossings with diploid wild species. Anther culture is the preferred technique for the rapid reduction of the ploidy level and has been successfully applied on different members of the *Solanaceae*. Cultivated potato, *Solanum tuberosum* is, however, an important exception.

In this study various potato genotypes (tetraploid cultivars, dihaploid breeding lines and a diploid wild species) were used in experiments concerning microtechniques, alternative culture methods and medium manipulation. The main objectives were to evaluate and compare the androgenetic ability of the various genotypes used and to try and identify the factors limiting their *in vitro* response.

Regarding *microtechnique*, the study focussed on the investigation of the frequency of androgenesis - as a function of plant age - and the determination of defined flower bud lengths representative of the correct microspore developmental stage for optimal androgenetic response. Combined with an extensive histological study on the microspore development within anthers, from the time of flower selection, after a cold-pretreatment and at various time-intervals during the culture period of 42 days, the following conclusions were reached: *In vitro* androgenetic response proved optimal when flowers of responsive genotypes were selected during the first seven to 21 days of the flowering period. Both microspore derived embryoid- and callus development were visible within responsive anthers after a culture period of only seven days. The flower bud length required for anthers to be in the optimal stage of microspore development, e.g. the uninucleate stage, varied between the different genotypes but could readily be determined with the DAPI (4,6-diamidino-2-phenylindole) technique. It was also concluded that anthers of the tetraploid cultivar Atzimba should be selected later, between the late-uninucleate and the early-binucleate developmental stages. This suggested a limited selection period for Atzimba anthers, as starch depositioning - which prevent embryogenesis - occurs within anthers during

the binucleate stage. Histologically, Atzimba showed limited embryoid development with no embryoid release, while the diploid wild species, *S. canasense*, proved androgenetically unresponsive.

Alternative culture methods were applied to study the effect of different culture phases (liquid, double layered and agar solidified) and anther orientations (lateral, dorsal and ventral) on the androgenetic response of the potato genotypes used. Liquid cultures, based on the so-called shed-pollen technique, enhanced the androgenetic response of the tetraploid cultivar Atzimba. Optimal embryogenesis was obtained for responsive breeding line 87.2002/3 with the utilization of agar solidified media, with maximal response when anthers were cultured in the lateral orientation. No response was observed from *S. canasense*.

The effect of *medium manipulation* on the androgenetic response of the three genotypes was investigated. The utilization of various combinations of different concentrations of indole-3-acetic acid (IAA) and benzyladenine (BA), the alteration of the initial time of incubation of anthers on the initiation media and the use of media without growth regulators compared to that containing gibberellic acid (GA_3), were investigated. BA had to be present in the initiation media and had a major, though not exclusive, effect on embryogenesis compared to IAA. The optimal BA concentration varied between the two trials. IAA also had an increasing effect on anther response, both in the absence of BA and, especially, in addition with relatively high BA concentrations. In this experiment, only breeding line 87.2002/3 responded. The initial culture of anthers, during the first seven to 21 days of the culture period, on media containing growth regulators proved essential for microspore derived embryoid production in the tetraploid cultivar Atzimba. As these growth regulators are metabolized in the culture media, the regular transfer at shorter, two-weekly intervals to media containing metabolically active substances, proved important. GA_3 had no enhancing effect on embryogenesis in any of the three tetraploid cultivars.

The results obtained in this study suggest that the **first 21 days** is the critical stage in the anther culture period in terms of the optimal time for flower selection, embryoid induction and the increase in embryogenetic response due to growth regulator influence. It is important to pre-determine the developmental stage when most microspores were in the uninucleate stage of development and to correlate this stage with a specific flower bud length. This would assure maximum response of those genotypes amenable to anther culture. It also implies a more practical and economical starting point to anther culture experiments. Following the determination of microspore developmental stage and pollen fertility, flowers should be selected from the donor plants only during the first three weeks of the flowering period. The

composition of the nutrient media used for potato anther cultures were sufficient with respect to growth regulators. The growth regulators BA, IAA and the amines glutamine and asparagine had to be present in the initiation media, especially during the first three weeks of the culture period. As microspore development within any one anther was found to be asynchronous, the regular transfer of anthers to fresh media is recommended to assure proper development of all microspores. The use of a slightly higher IAA concentration could be considered, but care should be taken as too-high concentrations would induce callus production. Microspore derived embryoid production is preferred, as the ploidy level of callus derived plantlets normally varies and somaclonal variation can occur. Liquid media should be considered for anther culture of tetraploid genotypes, while embryoid production can be increased by culturing the anthers of responsive genotypes on agar solidified media on the lateral orientation. Finally, the diploid wild species *S. canasense* seemed androgenetically unresponsive, or the media and culture conditions used did not satisfy the specific requirements of this genotype. Androgenetic amenability should first be transferred by means of interspecific crossings with a responsive dihaploid genotype, such as the breeding line 87.2002/3.

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DISCLAIMER

I hereby declare that the work presented in this thesis is my own. Where applicable, the work of others is acknowledged. I also declare that this thesis has not been submitted to any other University.

(Me) D. Liebenberg December 1995

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CHAPTER 1

LITERATURE REVIEW

I INTRODUCTION

Cultivated potatoes, as well as some 180 wild, tuber-bearing species, belong to the section *Petota* (formally *Tuberarium*); subsection *Potatoe* (formally *Hyperbasarthrum*) of the family *Solanaceae* (ROSS, 1986). According to reports the family *Solanaceae* consists of more than 84 genera and approximetely 3000 species (FERREIRA and ZELCER, 1988). They are divided into 18 series and all, except for a few, normally form stolons with terminally born tubers (ROSS, 1986). Most of the series consist of primarily diploid species (2n=24) (ROSS, 1986). The series *Tuberosa* comprises 68 wild and 8 cultivated species; most of which closely relate to *Solanum tuberosum* and possess a wide variety of resistances to pests, diseases, frost and other factors of economic importance (ROSS, 1986). The genus *Solanum*, consisting of approximetely 2000 species, is the biggest and most important member of this family. Edible species belonging to this family includes *S.melongena* (eggplant), *S. muricatum* (pepino) and *S. tuberosum* (potato) (FERREIRA and ZELCER, 1988).

Potato (*S. tuberosum* ssp. *tuberosum*) is one of the world's most valuable basic food crops, especially in terms of human consumption and nutritional value. It is superior to all other crops in protein production per unit area and time, and to most other crops in terms of energy production (Table 1) (ROSS, 1986).

Table 1 **The energy and protein production per day per hectare of some tropical food crops in all countries between 30°N.Lat. and 30°S.Lat.** ***(VAN DER ZAAG, 1976 - as quoted by ROSS, 1986)***

Crop	Energy production (10^3 kJ)	Protein production (kg)
Potatoes	166	1.1
Sweet potatoes	167	0.5
Cassave	154	0.2
Maize	133	0.8
Yams	121	0.6
Rice	120	0.6
Chick pea	67	0.9
Dry beans	40	0.6

When considering area under cultivation for human consumption alone, potato ranked 12th in the world in 1988; namely 17.86 million hectares (UGENT, 1970). According to ROSS (1986) consumption increased considerably and potato was ranked the 5th most cultivated crop in the world, while it occupied the fourth highest position in terms of yield, namely 312.2 million tons (Table 2). In general, consumption is enhanced and favoured by the plants great adaptability to different climates (VAN DER ZAAG and HORTON, 1983).

Table 2 **World production of the potato compared to other food crops in 1983 (FAO Production Yearbook 38, 1984 - as quoted by ROSS, 1986)**

Crop	Million hectares	Million tons
Wheat	231.9	521.7
Rice	147.5	469.9
Maize	129.6	449.3
Potato	20.3	312.2
Sweet potato	7.7	117.3
Sorghum	49.0	71.7

Cultivated potatoes are classified into four categories, based on the total number of complete sets of chromosomes contained in somatic cells (ploidy level) (UGENT, 1970):

(a) diploids - containing two complete sets (2n=24);

(b) triploids - containing three complete sets (2n=36);

(c) tetraploids - containing four complete sets (2n=48);

(d) pentaploids - containing five complete sets (2n=72).

II ARCHAEOLOGICAL HISTORY, DISTRIBUTION AND EVOLUTION

The most primitive potato fields, which signals the start of cultivation, were situated in South-east Peru and North-west Bolivia and date back 8000 years (ROSS, 1986). The oldest preserved remains of a potato was found in arhaeological excavations in Bolivia and in a cemetery at the coast of Chili (UGENT, 1970). These remains consisted of dried potato tubers and were discovered between 400 b.C. and 1000 a.C. (UGENT, 1970). Clay pots, resembling potatoes, were found in pre-Inca ruins, indicating that development of the cultivated potato occurred at this early stage in man's development (UGENT, 1970).

DISTRIBUTION AND EVOLUTION

The origin of the major crop plants is of great importance. Material from these sources provide useful genetic diversity as their wild relatives are often found in the centres of origin and evolution (HAWKES and FRANCISCO-ORTEGA, 1993). Most crops grown in Europe had their origin in other areas. From here they were introduced by travellers in ancient times (HAWKES and FRANCISCO-ORTEGA, 1993). Many crops were brought to Europe by, for instance Columbus, soon after the discovery of the New World in 1492. The potato, however, was not "discovered" until approximately 1532. This was mainly due to its origin in the high Andes of South America as it is a cool-temperate crop. The first

reference in the literature concerning the potato dates back to 1537 in Colombia (HAWKES and FRANCISCO-ORTEGA, 1993).

The domestication of the potato commenced with the migration of man from North-east Asia to Alaska during the Pleistocene, from where it spread further southwards and reached the Andes in Peru at 10 000 b.C. Tubers of wild potato species were among the first plants collected by nomads (UGENT, 1970). It was either accidental or intentional introduction that served as foundation for the early domestication of the potato (UGENT, 1970). Wild potatoes evolved to hybrid forms in the soil surrounding the semi-permanent camping grounds of nomads. These hybrids were distributed further and, as knowledge about the crop increased, more pleasant tasting, alkaloid-free diploid plants were selected.

The first introduction of tetraploid South American cultivars (Andigena type) was from the Magdalena Valley into Europe by the Spaniards. This took place in approximately 1570 (UGENT, 1970). These tubers probably originated from North Colombia (HAWKES, 1978). It represented a cool-temperate crop with adapted short-day characteristics (UGENT, 1970; ROSS, 1986). Consequently, it initially performed poorly in Europe until it acquired tolerance to long days. Evolution occurred and this can be traced by consulting herbarium specimens (ROSS, 1986). Such studies indicate that maturity now occurs earlier; that the number of secondary sprouts are reduced; that leaf dissection had increased; that stolons became fewer and shorter; tubers enlarged and developed a more acceptable shape and that overall yield increased. This proccess of evolution neared completion in 1750 and led to the development of the Tuberosum group (ssp. *tuberosum*) (ROSS, 1986). Introduction into Britain was most probably independent from that into Spain (UGENT, 1970). Potatoes are distributed worldwide except for the far-north and -south, with a high

concentration of species diversity in South- and Central America as well as Australia (UGENT, 1970). This distribution reflects the distribution pattern of the family (UGENT, 1970).

The first potato races were diploids from which triploids and tetraploids evolved (UGENT, 1970). No wild species are known which could be an ancestor of diploid wild cultivars. According to ROSS (1986) it could have disappeared by introgression and continous hybridization with semi-cultivated and wild forms. It was reported that the most important initial wild species, from which diploids readily evolved by means of spontaneous hybridization, included *Solanum canasense*, *S. leptophyes*, *S. soukupii* and *S. sparsipilum* (ROSS, 1986). Among these wild species, the *S. canasense/S. leptophyes* complex appears to be the most closely related to cultivated diploids (HAWKES, 1978; CHAVEZ, 1978 - as quoted by ROSS, 1986), while the *S. stenotomum/S. phureja* complex represents the most primitive of the diploid cultivated forms.

The transition from the cultivated diploid level to cultivated tetraploid species could occur by means of mitotic polyploidization or by the functioning of unreduced gametes (diplogametes) in both sexes of the same ór of different parental species such as *S. sparsipilum* (Figure 1) (ROSS, 1986). It is quite possible that tetraploid cultivars inherited their heterotic nature as a result of the combination of diplogametes representing genetically divergent groups (ROSS, 1986). Attempts to analyze *S. tuberosum*'s cytogenetic nature indicated that its inheritance followed a tetrasomic pattern which was in contrast with that of the wild tetraploid species (ROSS, 1986). Furthermore, some structural differences as well as the occurrence of many quadrivalents were found. After several studies, *S. tuberosum* was classified as an autotetraploid or a segmental allotetraploid (HAWKES, 1958 - as quoted by ROSS, 1986; HOWARD, 1973; WOODCOCK and HOWARD, 1975; RICKEMAN, SHARON and DESBOROUGH, 1978).

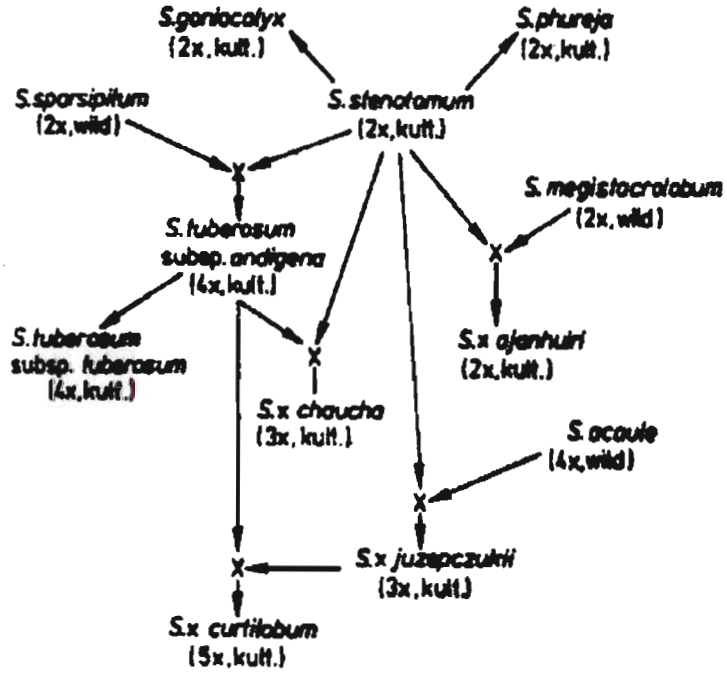


Figure 1 The origin of cultivated potato species *(HAWKES, 1981)*

According to UGENT (1970) permanent gene flow exists from wild species via hybrids into cultivated forms. This confirms the general idea that the evolution of the cultivated potato in its gene centre is a continuous process (ROSS, 1986).

III CLASSIFICATION

Differences occur concerning the number of distinctive species of cultivated potatoes. Dodds (1962) classified *S. tuberosum* as the only cultivated species and *S.* x *juzepozukii* and *S.* x *curtilobum* as hybrid cultivars (UGENT, 1970). According to other botanists there are as many as 20 cultivated species. The classification system of DODDS (1962) is presented in Table 3. This is a unique system in that it is the only system following the International Code of Nomenclature of Cultivated

Plants. Other systems were constituted according to the ordinary International Code of Botanic Nomenclature which was developed primarily for wild species application (UGENT, 1970).

Table 3 Potato classification system of DODDS (1962) *(from UGENT, 1970)*

Species	Distribution	Origin
S. tuberosum 1. Group Tuberosum (2n=48)	cosmopolitan	by artificial selection in Europe, North America and Chili from introduced clones of group 2
2. Group Andigena (2n=48)	Venezuela to northern Argentina; sporadically in Central America and Mexico	from groups 4 & 5 by spontaneous doubling of the chromosome number
3. Group Chaucha (2n=36)	Central Peru to northern Bolivia	by hybridization between groups 2 & 4, or 2 & 5
4. Group Phureja (2n=24)	Venezuela to northern Bolivia	by selection for short tuber dormancy from group 5
5. Group Stenotomum (2n=24)	Southern Peru to northern Bolivia	by natural hybridization between wild species followed by artificial selection
S. x *juzepozukii* (2n=36)	Central Peru to southern Bolivia	from crosses of *S. acaule* with group 4 or 5
S. x *curtilobum* (2n=60)	Central Peru to southern Bolivia	crosses of *S.* x *juzepozukii* (unreduced) with group 2

IV POTATO CYTOLOGY

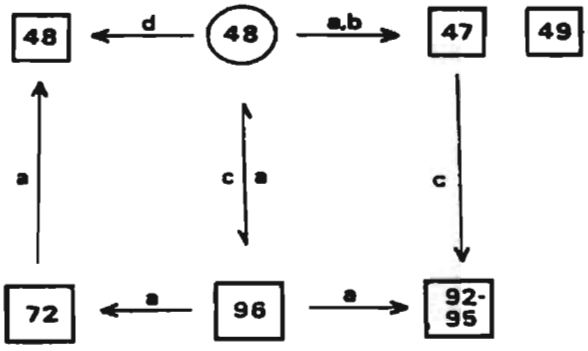
1. The basic chromosome number

The genus *Solanum* has a basic chromosome number of 12 (ROSS, 1986). *S. tuberosum* ssp. *tuberosum* is, however, an autotetraploid with chromosome number 2n=4x=48. Based on their different chromosome numbers (ploidy levels), potatoes are divided into different groups (as discussed in the *INTRODUCTION*).

2. Chromosomal and Phenotypical Variations

During meiosis both univalent and multivalent chromosomes can occur in different potato species. This leads to the production of gametes with either one (or more) chromosome fewer ór more than the euploid chromosome number (UGENT, 1970). For example, in a study with 700 individual *S. tuberosum* plants, one plant was found with a chromosome number 2n=46; one with 2n=47 and one with 2n=49 (HOWARD, 1978). In another study with 36 *S. tuberosum* plants regenerated from protoplast-mesophyll cells, 61% maintained their parental chromosome number of 2n=48, while the remaining 39% had chromosome numbers varying from 28% octaploids (2n=76), 3% hexaploids (2n=72), 6% monosomic (2n=47) and 5-6% trisomic individuals (2n=49) (HOWARD, 1978).

Figure 2 indicates how these chromosomal and phenotypical variations could occur. Octaploids probably resulted by means of endomitosis[(c)], while aneuploids could result from nucleus-fragmentations[(a)] and chromosomal nondisjunction[(b)] (mitotic abnormalities). Furthermore, equational nondisjunction[(d)] could also occur, leading to segregation and expression of phenotypic variation without altering the initial chromosome number (GILL, KAM-MORGAN and SHEPARD, 1986).



nucleus fragmentations[(a)]; chromosomal non-disjunction[(b)]; endomitosis[(c)]; equational non-disjunction and mutations leading to structural changes in chromosomes[(d)]

Figure 2 Origin of potato chromosomal variation *(GILL, KAM-MORGAN and SHEPARD, 1986)*

3. Somatic Chromosomes and their Evolution

The somatic chromosomes of *Solanum* species are small and difficult to detect and study. HOWARD (1978) found that somatic chromosomes of the two tetraploids *S. tuberosum* and *S. longipedicellatum* could successfully be studied, following 8-hydroxyquinoline treatment. He distinguished seven different groups of somatic chromosomes with satellites. *S. longipedicellatum* is an allotetraploid and *S. tuberosum* an autotetraploid which lost its two satellites during evolution from their diploid ancestors (HOWARD, 1978). Chromosomal evolution of higher plants is studied mainly by means of meiotic analyses and karyotype comparisons. When studying the chromosomal evolution of the potato by means of cloning and RFLP-mapping, BONNIERBALE, PLAISTED and TRANKSLEY (1988) found that alterations to the basic potato chromosome were mainly caused by chromosomal translocations. However, since the evolution of the potato and tomato (family *Solanaceae*) from their mutual ancestor, only paracentric inversions occurred. This normally led to

alterations in gene-sequence on the chromatids, without altering chromosome number or chromatid-ratios (BONNIERBALE, PLAISTED and TANKSLEY, 1988).

V GENETICS AND BREEDING

1. Conventional Potato Breeding

The main objective of breeding is to increase yield; maintain good quality and to improve resistance to pests and diseases. Furthermore, the exploitation of heterosis (i.e. when the progeny surpasses the value of the best parent's vigour and yield) is the most important goal in practical potato breeding (ROSS, 1986). According to HAWKES (1979), GLENDINNING (1979) quoted by ROSS (1986) and ROSS (1979) quoted by ROSS (1986), the genetic base of potato requires constant broadening by means of breeding. Continuous improvement is of great importance especially for potato which is the third most cultivated crop in South Africa, but does not easily adapt to local conditions due to its original cool-temperate origin. In view of classical genetic considerations the potato is a complex crop due to its tetraploid nature, consequent tetrasomic inheritance patterns, the crop's limited genetic base and high degrees of heterozygosity and self-incompatibility. Due to these barriers, classical breeding methods and propagation of the crop by means of seed for overall crop improvement, is a slow process.

ROSS (1986) stated that more than 50 traits should be combined in a modern variety. In order to breed an improved variety, an enormous number of seedlings have to be raised annually, as most traits are inherited by polygenes and only a few seedlings express traits to the same degree as the parent(s). According to ROSS (1986) it takes six to eight years to screen these seedlings and to consequently select the most superior genotypes. These superior genotypes have moderate

expression of most traits and a more than average expression in only a few. LIU and DOUCHES (1993) also indicated that after selecting promising clones from these bulk F_1-hybrid seedlings, the latter have to be vegetatively propagated and performance tested for at least seven to ten years. This also implies increasing plot sizes and locations and thus an overall decrease in cost-effectiveness.

Successful and efficient breeding mainly relies on the selection of the best parents, which will produce seedlings with valuable traits and a heterotic nature (ROSS, 1986). The less genetically related the parents, the bigger the chance to obtain an increased heterotic character. Thus, for maximum utilization of heterosis, potato breeding requires the selection and crossing of highly homozygous parents. These homozygous parents must comprise maximum combination of favourable genetic traits, which would ensure sufficient recombination and increasing heterozygosity (tetra-allelism is the main source of heterosis) (ROSS, 1986). Attempts were previously made to obtain homozygous parents by means of several generations of inbreeding. But, due to the self-incompatible nature of *S. tuberosum* and most cultivated diploid potato species for that matter, inbreeding-depression occurred (ROSS, 1986). This is due to the crop's outbreeding character and led to a decrease in plant size, yield and vigor. Self-incompatibility implies a pre-zygotic mechanism which is common in both wild and cultivated diploid potato species; while one- or two-way incompatibility (S-locus systems) proved characteristic of the family *Solanaceae*; in particular *S. tuberosum* (ROSS, 1986; KIRCH, UHRIG, LOTTSPEICH, SALAMINI and THOMPSON, 1989). Self-incompatibility can be overcome by the doubling of the chromosome number, but it will be re-established in dihaploids extracted from the tetraploids (ROSS, 1986). The latter is essential to keep in mind when diploid self-incompatible species are used as female parents in crosses with diploid self-compatible species (ROSS, 1986). Inbreeding depression was found to vary between both

potato families and different traits (ANON, 1985 - as quoted by ROSS, 1986). This implies that it is possible to select for tolerance to inbreeding (ROSS, 1986).

Crossing techniques can be performed either outdoors or in the greenhouse, but both temperature and light are crucial factors. Temperature must remain below 20-22°C, while continuous light in the greenhouse is essential (ROSS, 1986). Another crucial factor is the handling and storing of the pollen used from the male parents. Overall, conventional crossing techniques require great care and specific conditions which cannot always be controlled and maintained due to unforseen environmental influences.

Potatoes are, as mentioned earlier, highly heterozygous and cross-pollination between tetraploid potato clones would result in a progeny which displays complex segerations of agronomic important characteristics, pest and disease resistance and other traits (LIU and DOUCHES, 1993). Consequently, it is difficult to study the inheritance of economic important traits in cultivated potatoes. LIU and DOUCHES (1993) concluded that genetic studies could be simplified by haploid production. This could be accomplished by reducing the ploidy level from the tetraploid to the diploid level, followed by another reduction cycle to obtain the haploid condition (LIU and DOUCHES, 1993). According to CHASE (1963) potato breeding based on crossings of complementary haploid genotypes and selection at the diploid level, are much more simple and efficient compared to traditional tetraploid crossing-approaches.

Both wild and primitive cultivated potato species are of great importance in conventional breeding schemes, as they provide a broad spectrum of gene-pools (ROSS, 1986). They consequently possess a wide variety of different traits; especially resistance to pathogens, pests and disease;

which make them extremely valuable in breeding for heterotic genotypes (ROSS, 1986). In attempting to extend the crop's genetic base, cultivars of the ssp. *andigena* have had an important impact, as it was recently illustrated that most traits found in wild species could be traced to primitive cultivars as well (ROSS, 1986). With the implementation of cultivated species, less backcross generations were required (ROSS, 1986). Although crosses between two tetraploid *S. tuberosum* subspecies is a normal procedure, RASCO, PLAISTED and EWING (1980) concluded that two to three backcrosses to ssp. *tubesosum* are necessary for adaption to long days. This also results in increased heterosis. According to ROSS (1986) the diploid cultivated *S. phureja* showed good combining ability when crossed with ssp. *tuberosum*. *S. phureja* furthermore produce unreduced gametes which transmitted almost the complete valuable genotype to the tetraploid progeny after a 4x-2x-cross (ROSS, 1986). This enhanced heterosis. *S. phureja* pollen also induce the process of parthenogenesis (see section 2.1) in tetraploids (ROSS, 1986).

When considering incorporating wild species in a breeding program, breeders have to keep in mind that some diploid wild species cannot be crossed directly with *S. tuberosum* due to the large differences that occur between wild and cultivated species (ROSS, 1986). These differences are caused by various "isolating mechanisms" such as eco-geographical separation, a difference in ploidy number, different flowering times, genome differences and a difference in endosperm balance number (EBN) (HAWKES, 1990). For instance, those species with an EBN = 1 will not cross and produce hybrids with those with EBN = 2, even if the species occur within the same ecological zone (HAWKES, 1990). Consequently, breeders have to attempt to overcome these barriers in order to make agronomic and economic important genes accessible for incorporation. This, however, requires repeated backcrossings and testing of progeny which is extremely time-

consuming. A further drawback is that the polygenically determined characters may be lost as a result of these repeated backcrossings. It was consequently suggested at the CIP workshop in January 1973, that cultivated forms should receive first priority. Some combination success has been obtained between *S. bulbocastanum* (resistant to late blight) via *S. acaule* as a "bridge species" with *S. phureja*; the latter being compatable with *S. tuberosum* (ROSS, 1986).

Direct hybridization of tetraploid wild species such as *S. acaule* and *S. stoloniferum* with ssp. *tuberosum* is seldom successful, due to the hampering of endosperm development (ROSS, 1986). This can be overcome by, for instance, spontaneous formation of unreduced egg cells by the wild species ór by colchicine treatment (ROSS, 1986). In general, initial hybridization with *S. tuberosum* must be followed by several generations of backcrossing (as discussed previously) to establish euploidy in appropriate cases; to achieve general climatic adaption-ability and to eliminate undesirable genes (wild characteristics such as small tubers, etc.) (ROSS, 1986). The general question that arise is whether it would be best to first combine different wild species, followed by a single line of backcrossing; ór to first build up separate back-crossed lines and then eventually combine them (ROSS, 1986)?

2. Breeding at the Diploid Level

The necessity for the development of rapid, more efficient breeding techniques soon became evident due to the problems associated with conventional breeding at the tetraploid level. Haploids (see section 2.3) became the obvious means of circumventing these problems, especially simply-inherited traits (LIU and DOUCHES, 1993). The importance of the reduction of the ploidy level (from tetraploid via dihaploid to the monohaploid level) for breeding of genetically improved clones was first emphasized by CHASE (1963). According to ROSS (1986) the three main trends that characterize modern development in potato breeding

comprise: (a) the recognition of tetra-allelism as a main source of heterosis; (b) dihaploid and monohaploid production as a way to systematically construct tetra-allelic genotypes; and (c) the by-passing of meiosis. The production of dihaploids from tetraploid potato cultivars and breeding lines can be obtained either parthenogenetically ór by means of anther cultures (androgenesis) (SOPORY and BAJAJ, 1987).

2.1 Parthenogenesis

Parthenogenetic dihaploid production by means of interspecific crosses of tetraploids with *S. phureja* clones (pseudogamy) became a routine procedure (ROSS, 1986; LIU and DOUCHES, 1993) following the identification of *S. phureja* (2n=2x=24) as a superior diploid pollinator for the induction of parthenogenesis in potato (HOUGAS and PELOQUIN, 1958; HOUGAS, PELOQUIN and GABERT, 1964). *S. phureja* clones were consequently developed and utilized as haploid inducers in order to facilitate identification of hybrids at the seed stage. This aspect of haploid induction is based on the development of an unfertilized egg cell. However, different cytogenetical groups of seedlings can develop from tetraploid x *S. phureja* crosses (ROSS, 1986).

S. phureja carries a dominant seedling marker "P" for purple hypocotyl and, when homozygous, facilitates the identification of haploids (pp) in interspecific progeny (LIU and DOUCHES, 1993). A purple spot appears at the base of the cotyledons of the embryo, which is visible on both sides of the seed (LIU and DOUCHES, 1993). This purple colouration is also apparent at the base of all plant organs; thus it serves as a nodal marker (LIU and DOUCHES, 1993). The absence of the purple spot indentifies potential dihaploids but unfortunately the spot is not always clearly distinguishable. CALIGARI, POWELL, LIDDELL, DE MAINE and SWAN (1988) declared that the appearance of the spot can vary with the genetic background. Consequently, misclassification can occur. This relative easy detection of haploids was of great importance especially as

haploids occur in relatively low frequencies in *S. tuberosum* following interspecific crosses (HOUGAS, PELOQUIN and ROSS, 1958).

After selection of the spotless seed, germination is stimulated by gibberellic acid and planted in seed trays. When plantlets are sufficiently strong they are transplanted into pots in the glass house. After a few weeks, a provisional check of the chromosome number can be made by counting the chloroplasts in the gaurd cells of the stomata (ROSS, 1986). The number of chloroplasts correlates with the chromosome number (ROSS, 1986). Chloroplast numbers between 10 - 14 correlates with the diploid level, between 15 - 20 with the triploid level and counts between 20 - 25 with the tetraploid level.

2.2 Androgenesis

The production of dihaploids from tetraploids by means of pseudogamy made it possible to perform pre-breeding at the diploid level (ROSS, 1986). A second reduction in chromosome number by means of anther culture (androgenesis) can be performed on regenerated dihaploids, leading to the production of monohaploids (section 2.3). The utilization of specialized tissue culture techniques to obtain these monohaploids, gave new impetus to modern plant breeding. It not only increased variation, but also extended the germplasm-pool. Although anther culture techniques can be utilized for both dihaploid and monohaploid production, more success has been obtained in haploid production in several crops (JOHANSSON, 1986; MORRISON and EVANS, 1988). The success of anther culture depends upon the fact that microspores are cultured *in vitro* while it is nourished and protected *in vivo* within the anthers until maturity (JOHANSSON, 1986). *S. tuberosum* is, however, an important exception and complete dihaploid potato clones have been derived from only a few tetraploid genotypes.

Selected flower buds receive a cold pretreatment, are surface sterilized,

anthers are dissected under aseptic conditions from the buds and plated on a specific formulated nutrient medium. Cultures are then incubated and remain in culture for about three months. Developed embryoids are harvested and transferred to an embryoid-regeneration medium after which plantlet development is monitored and consequent determination of ploidy level of regenerated plantlets is conducted.

In order to achieve an optimum androgenetic response, different anther culture methods and medium manipulation techniques have been developed and its effect on tetraploid potato cultivars, diploid wild species and dihaploid breeding lines will be determined in this study. ZAPATA, TORRIZO, ROMERO and ALEJAR (1982) also stressed the importance to study the best cultural and physiological condition for triggering androgenesis as only a low frequency of callus production and plantlet regeneration was observed in *Oryza sativa*.

2.3 The Production and Utility of Haploids

As mentioned previously, haploids serve as an alternative to overcome problems associated with complex tetraploid potato breeding. However, the occurrence of natural haploids of higher plants are rare and restricted to only a few plant species (KIMBER and RILEY, 1963). The experimental induction of haploidy in general is unsuccessful for most species and only a few haploid individuals have been produced (VASIL, AHUJA and VASIL, 1979). Consequently, many attempts were made since the 1950s to induce haploid production from haploid gametophytic cells. According to VASIL, AHUJA and VASIL (1979) haploid tissue cultures were obtained from the pollen of several gymnosperm species such as *Ginkgo biloba* (TULECKE, 1953, 1957), *Taxus* (TULECKA, 1959), *Torreya nucifera* (TULECKA and SEHGAL, 1963) and *Ephedra foliata* (KONAR, 1963). However, no shoots or plantlets developed from these haploid tissue cultures. Early work on anther cultures of angiosperms was done by VASIL (1967, 1973) mainly to obtain a better understanding of the

meiotic process (VASIL, AHUJA and VASIL, 1979). YAMADA, SHOJI and SINOTO (1963) reported the first production of microspore derived haploid angiosperm tissue cultured from anther cultures of *Tradescantia reflexa*. These culture did not undergo organogenesis (VASIL, AHUJA and VASIL, 1979). The initial success on haploid plant production resulted from the work by GUHA and MAHESHWARI (1964). Haploids were obtained by means of anther culture performed on *Datura innoxia* (MAHESHWARI, RASHID and TYAGI, 1982). Anther cultures of this plant, developed from the pollen grains, resulted in haploid embyoids (GUHA and MAHESHWARI, 1966,1967).

Following this initial breakthrough, many researchers were inspired to induce haploids in other plants as well, and intensive studies were extended to *Nicotiana* by BOURGIN and NITSCH (1967 as quoted by VASIL, AHUJA and VASIL, 1979), NAKATA and TANAKA (1968 as quoted by VASIL, AHUJA and VASIL, 1979) as well as MAHESHWARI, TYAGI and MALHORTA, (1980). BOURGIN and NITSCH (1967 - as quoted by VASIL, AHUJA and VASIL, 1979) obtained mature, flowering haploid plants from anther cultures of *Nicotiana tabacum* and *N. sylvestris* (VASIL, AHUJA and VASIL, 1979). According to MAHESHWARI, RASHID and TYAGI (1982) androgenesis has been reported in more than 171 species, including hybrids, belonging to some 60 genera and 26 families of angiosperms.

During meiosis each gene is represented once as only half of the chromosome number occurs. When immature anthers of a diploid plant are cultured *in vitro* the gametic cells (microspores) are stimulated to divide symetrically and eventually give rise to a plant (sporophyte). This is in contrast to gametic cells *in vivo* which would develop to produce the male gametopyte, the sperm cell. A microspore derived plant would therefore contain half the chromosome number of the donor plant and would be haploid. The single chromosome set of a haploid plant can then be duplicated to produce a completely homozygous, doubled

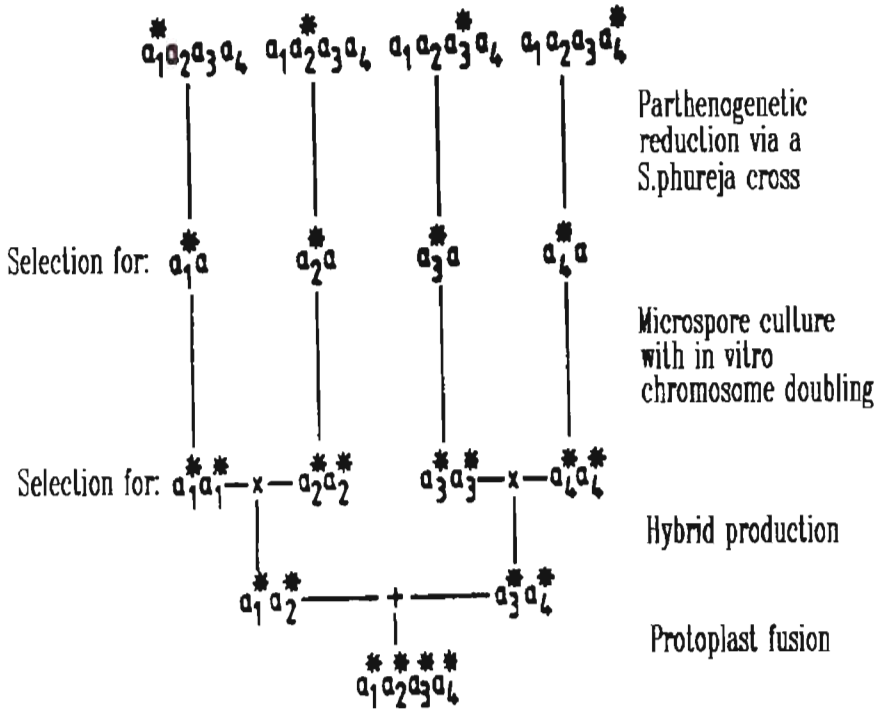
haploid (DH) which is equivalent to a stable line produced by means of inbreeding. Thus, haploids are of major importance as they are the only source for the production of large numbers of fertile, homozygous dihaploid clones by means of diploidization and clonal propagation in only one generation (VASIL, AHUJA and VASIL, 1979; SOPORY and BAJAJ, 1987; MORRISON and EVANS, 1988). This provides a much faster method, compared to conventional inbreeding methods, for the production of homozygosity in self pollinating species. Furthermore, the utilization of haploids simplifies breeding of higher plants, due to the fact that recessive traits are not masked by dominant genes in the haploid condition. Consequently all genetic traits are expressed at this breeding level. This also leads to the early detection of mutations of scientific and economic importance. These mutations are, due to their recessive nature, normally difficult to detect in heterozygous plants.

Haploids also present a rich source of genetically different genotypes which can be submitted to selection (ROSS, 1986). Due to haploid breeding, less back-crossings are neccessary for the removal of undesirable traits; desirable traits can be combined using smaller populations and selections are simplified since all traits are expressed.

SOPORY AND BAJAJ (1987) proclaimed that potatoes are quite amenable to tissue culture techniques such as monohaploid production via anther cultures; the culture and regeneration of protoplasts from tetraploid and dihaploid clones and the possibility of protoplast fusion within certain *S. tuberosum* clones and species. WENZEL (1994) ascribed this amenability to the crop's clonal propagation features and proclaimed that it has "under physiological aspects of cell culture research an outstanding position as a *Solanaceaous* crop". Considering the possible utilization of these mentioned techniques in potato breeding, WENZEL (1979 as in SOPORY and BAJAJ, 1987) proposed an analytical synthetic breeding scheme (Figure 3). According to this scheme the reduction of the

tetraploid level of cultivated potatoes to the dihaploid level (2n=2x=24) is suggested (of at least four different clones, each of which contains some positive, qualitatively inherited characters like disease resistance, ect.) and eventually to the monohaploid level (2n=x=12). Selection of preferably a dominant character, followed by chromosome doubling, can be conducted at the 2x level, leading to the production of homozygous plants (SOPORY and BAJAJ, 1987). Heterozygous diploids resulting from subsequent sexual intercrossings, can again undergo protoplast-fusion to produce tetraploid hybrids derived from four different haploid genomes (MEYER, SALAMINI and UHRIG, 1992). After each stage selections, either *in vitro* or conventional, must be conducted to obtain favourable genotypes (ROSS, 1986). It is very important that the ploidy level of produced dihaploids should be re-established to the tetraploid level, especially since maintaining high levels of heterozygosity is such an important factor (SOPORY and BAJAJ, 1987). These synthesized tetraploids can be utilized further as parents in conventional breeding (CHASE, 1963). Resynthesis of the tetraploid level can be accomplished in several ways (section 2.5).

In experiments conducted by WENZEL and UHRIG (1981), WENZEL's scheme was utilized, mainly to produce homozygous dihaploid clones containing resistance against potato virus X, Y and PLRV and resistance to potato cyst nematodes. This resulted in successful selection of resistant androgenetic clones and these resistances followed both qualitative and quantitative modes of inheritance (WENZEL and UHRIG, 1981). By means of anther culture, doubled monohaploid clones were obtained showing resistance in the homozygous state. Resistance could be observed in subsequent field trials as well (SOPORY and BAJAJ, 1987).



a = Potato genome
** = Favourable genes with qualitative inheritance (e.g. resistance)*

Figure 3 Analytical breeding scheme for potato improvement by means of combination breeding *(WENZEL et al., 1979)*

2.4 Combination Breeding

Despite all the advantages of unconventional breeding, the latter can only be successful when integrated in an ordinary, conventional potato breeding scheme (ROSS, 1986). This was successfully illustrated with WENZEL's breeding scheme (section 2.3). According to ROSS (1986) dihaploids and monohaploids have to be extracted from, and gene transfer applied to, top combinations or varieties resulting from classical breeding. Consequently, classical breeding is considered a precondition

which must be performed at both the tetraploid and diploid level (ROSS, 1986). WENZEL (1994) also emphasized the importance that unconventional tissue culture techniques must be accompanied by, and combined with, classical breeding methods, regardless whether selection or gene-combination were concerned. SOPORY and BAJAJ (1986) proposed a combination of conventional and unconventional breeding techniques. This would imply a combination of parthenogenesis, androgenesis, protoplast fusion and classic combination breeding techniques. It comprises dihaploid production from the tetraploid level by means of parthenogenesis, consequent monohaploid production by means of androgenesis and the development of homozygotic diploids through chromosome doubling which normally occurs spontaneously during the culture period.

2.5 Resynthesis of Tetraploids

Neither diploids nor dihaploids can fulfil all the requirements of a modern variety (ROSS, 1986). As mentioned the resynthesis of tetraploids from the dihaploid level is essential (SOPORY and BAJAJ, 1987). This can be achieved by means of (a) somatic polyploidization; (b) meiotic polyploidization; or (c) somatic hybridization.

(a) Somatic/Mitotic Polyploidization

This form of chromosome doubling can be induced chemically with colchicine. Mitotically induced tetraploids, however, do not perform as well as their diploid parents (ROSS, 1986). They also have decreased pollen fertility. Colchicine utilization is therefore discouraged.

(b) Meiotic Polyploidization by means of Diplogametes

This type of chromosome doubling relies on the application of unreduced (2n) (diplogametes) which result from deviations from the normal meiosis mechanism (ROSS, 1986). A female tetraploid, or a female diploid producing unreduced gametes, can be used in crossings

with a male diploid parent that produces unreduced pollen. This lead to the development of an inbred progeny, consisting of triploids and tetraploids. These tetraploids contain high levels of inbreeding even if the diploid parents are not related (ROSS, 1986). With related, inbred parents, the coefficient of inbreeding increases (F-value) while heteroses decreases. Unreduced gametes play an important role in the conservation of genotypes despite inbreeding.

(c) Somatic Hybridization/Protoplast Fusion

Interspecific protoplast fusion forms an integral part in potato breeding programs. Somatic hybrids play an important role in potato improvement and can be produced by means of successful protoplast fusion techniques. Hybrids can be produced from sexual incompatable lines ór from lines where male and female sterility prevented crossings due to the fact that somatic hybridization is independant of gametes (ROSS, 1986). Somatic hybrids are also valuable where parents are sexually compatable. During crossings, only female cytoplasm is transferred, while the cytoplasm of both parents fuse during somatic hybridization.

Of the three methods described above, somatic hybridization is the most important since both the self-replicating organelles and dominant inherited traits of both parents can be combined in the hybrid (ROSS, 1986). Hybrids differ from both the parents as well as from sexual hybrids concerning heterotic development and resistance to potato virus Y (ROSS, 1986).

VI UTILIZATION OF ANTHER CULTURES IN DIPLOID BREEDING

1. Microsporogenesis

The process of microsporogenesis comprises the development

sequence of mature pollen grains (male gametophytic generation of higher plants) from the spore mother cells (SUNDERLAND, 1974). This development can be divided into three phases: (1) The meiotic process and the formation of spore-tetrads; (2) dissociation of tetrads and the development of individual microspores (pollen contains unicellular microspores at this stage); (3) maturation of pollen grains from the microspore stage (pollen are here in the multicellular gametophytic stage or "pollen grains") (SUNDERLAND, 1974). In Angiosperms the microspore mother cell undergoes meioses, leading to the formation of four haploid microspores. This signals the begin of the gametophytic phase in the life cycle of Angiosperms (VASIL, AHUJA and VASIL, 1979). These haploid microspores are enclosed within a callose (B-1,3-glucan) tetrad wall which is later dissolved by an enzyme (callase or B-1,3-glucanase), synthesized by the somatic anther wall (VASIL, AHUJA and VASIL, 1979). The four microspores are now released and haploid individuals can develop from these isolated microspores in many different ways.

The general mode of microspore development (usually under *in vivo* conditions) results in two male gametes, a tube nucleus and a pollen tube (Figure 4, far left [a]). A vacuole pushes the nucleus of the microspore to one end of the spore and "microspore mitosis" (or the "first pollen mitosis") follows with the nucleus situated in this position (SUNDERLAND, 1974). Two unequal cells are formed by this so-called asymmetric mitosis (type A pollen grain). A cell-plate divides the daughter- or generative nucleus from the vegetative nucleus. The larger vegetative nucleus contains diffuse chromatin and becomes highly metabolically active, leading to the production of the pollen tube. The smaller generative nucleus with condensed chromatin undergoes another mitotic division, forming the two male gametes (sperms). In *Nicotiana*, which is considered a typical representative of the Angiosperms, pollen is normally shed in this bicellular condition (SUNDERLAND, 1974). Several possibilities exist following the *in vitro*

culture of isolated microspores (DODDS and ROBERTS, 1985). Firstly, the mechanism which causes asymmetry during first pollen mitosis breaks down. The nucleus is now situated more to the centre of the microspore and consequent mitosis results in normal symmetrical (SUNDERLAND, 1974) or "symmetrical microsporogenesis" (PIERIK, 1993). This so-called B type pollen grain contains two similar* or equal cells (Figure 4) (DODDS and ROBERTS, 1985). These similar nuclei can either undergo repeated divisions, producing a haploid embryoid (Figure 4, second from right [b]) or they can fuse (Figure 4, far right [c]), producing a diploid embryoid. Secondly, the generative and vegetative nuclei are formed as described according to the *in vivo* development sequence, from where several *in vitro* development patterns could lead to the production of haploid embryoids. In the first instance, the vegetative nucleus can divide repeatedly, producing a haploid embryoid while the generative nucleus disintegrates[d] (DODDS and ROBERTS, 1985; PIERIK, 1993). This is the most frequently occurring phenomenon *in vitro*. Alternatively, the generative nucleus divides into a haploid embryoid while the vegetative nucleus disintegrates[e] (DODDS and ROBERTS, 1985; PIERIK, 1993). The latter is of unusual occurrence and is observed in *Hyoscyamus* (PIERIK, 1993). Furthermore, haploid callus tissue[f] can develop from microspores, forming embryoids *de novo* (DODDS and ROBERTS, 1985).

Due to all these possible developmental events which could lead to embryoid formation, considerable variation can occur in resulting ploidy levels. It is therefore of major importance to determine the ploidy level (chromosome number) of all newly developed plantlets.

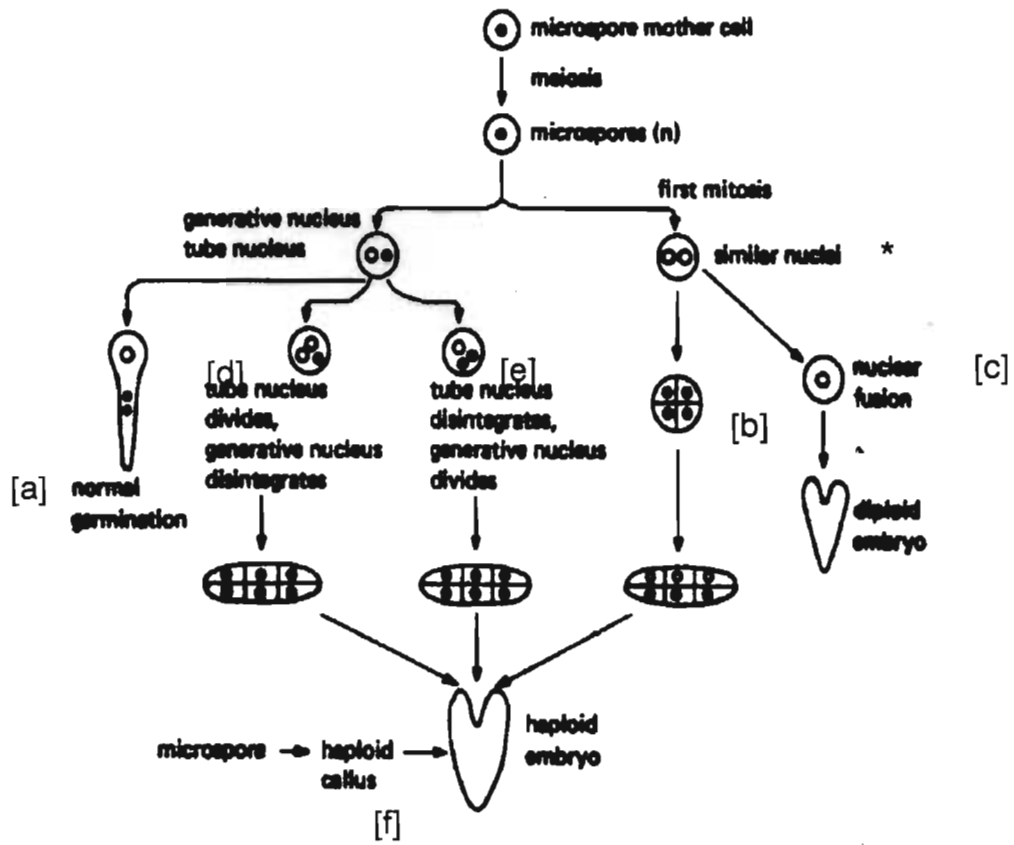


Figure 4 *In vivo* and *in vitro* microspore developmental pathways *(DEVREUX, 1970 from DODDS and ROBERTS, 1986)*

2. Culture Methods

When considered from both a genetic and a plant breeding view, tissue culture techniques and haploid plant production are advantageous for overall crop improvement. Several methods for *in vitro* haploid plant production and utilization were consequently developed. According to KELLER and STRINGAM (1978), most efforts were devoted to *in vitro* haploid production from immature pollen grains (microspores) and the process of androgenesis became by far the most important method to induce *in vitro* regenerated haploids. This is of importance due to the fact that considerable numbers of pollen grains can all be developed

separately into haploids (PIERIK, 1993). Microspore derived plants were obtained by culturing anthers (a) on solid medium (GUHA and MAHESHWARI, 1964); (b) floating on liquid medium (MAHESHWARI, RASHID and TYAGI, 1982); (c) by culturing whole inflorescences in agitated liquid medium (PIERIK, 1993; WILSON, MIX and FOROUGHI-WEHR, 1978) or (d) by culturing isolated microspores (KELLER and STRINGAM, 1978).

2.1 Isolated Pollen Cultures

Several researchers investigated the success of isolated pollen culture on different species. According to MAHESHWARI, TYAGI and MALHORTA (1980) researchers such as NIIZEKI and GRANT (1971); BINDING (1972); SHARP, RASKIN and SOMMER (1972) reported the occurrence of divisions in isolated pollen grain cultures although no complete developed embryoids resulted from any of these investigations. In 1973, NITSCH and NORREEL successfully induced defined embryoids from isolated *Datura innoxia* pollen grains (MAHESHWARI, TYAGI and MALHORTA, 1980). This was obtained through the so-called *"ab initio"* pollen culture technique which concerns the isolation of pollen by gently macerating the anthers with a pestle in liquid medium. This was followed by filtering the microspore suspension through a sieve and washing it with medium by centrifugation (MAHESHWARI, TYAGI and MALHORTA, 1980). Isolated pollen was then plated on an appropriate medium. According to MAHESHWARI, TYAGI and MALHORTA (1980) SANGWAN and NORREEL (1975) extended this technique to *Petunia hybrida*, while WENZEL, HOFFMANN, POTRYKUS and THOMAS (1975) and SOPORY (1977) cultured pollen grains of *Secale cereale* and *Solanum tuberosum* respectively. In all later attempts only a few, if any, divisions occurred, while the frequency of responding *Petunia hybrida* pollen grains was extremely low (MAHESHWARI, TYAGI and MALHORTA, 1980).

In order to increase response, several attempts were made to modify existing techniques. Anthers were precultured for a few days before

isolation of pollen in liquid medium. Pollen was then either plated in the same medium or processed further as in *ab initio* pollen culture. It was reasoned that initial pre-culturing within the anther would provide a more stable environment for the critical phase of the initiation of androgenesis; with resulting pollen grains being much more amenable to culture procedures. This modification led to an increase in response when applied to anthers of *Nicotiana tabacum* (NITSCH, 1974a - as quoted by MAHESHWARI, TYAGI and MALHORTA, 1980); REINERT, BAJAJ and HEBERLE, 1975; WERNICKE and KOHLENBACH, 1977), *Solanum tuberosum* (WEATHERHEAD and HENSHAW, 1979 - as quoted by MAHESHWARI, RASHID and TYAGI, 1982) and *Oryza sativa* (CHEN, WANG, TIAN, ZUO, ZENG, LU and ZHANG, 1980).

Further refinements of pollen culture techniques soon followed; one of which was developed in 1977 by SUNDERLAND and ROBERTS for *Nicotiana tabacum* (MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982). This "serial culture technique" was based on the so-called shed pollen phenomenon, where anthers cultured in liquid medium dehisce and shed their pollen into the medium. Anthers were then transferred to fresh liquid medium at intervals. During each culture period anthers shed pollen into the medium which led to the development of a series of cultures of "free" pollen (MAHESHWARI, TYAGI and MALHORTA, 1980). This free pollen continued to develop into embryoids. WENZEL and his coworkers (1975) and WERNICKE, HARMS, LÖRZ and THOMAS (1978) further refined existing pollen culture techniques using *Secale cereale* and *Nicotiana tabacum*. In both attempts an enriched fraction of potentially embryogenic (viable) pollen grains were separated from the general population of isolated pollen of precultured anthers. This could be accomplished by one of two possible methods. Firstly, by layering the filtered pollen suspension over a highly concentrated sucrose solution followed by centrifugation, or secondly by density gradient centrifugation (MAHESHWARI, TYAGI and MALHORTA, 1980;

MAHESHWARI, RASHID and TYAGI, 1982). In the first instance the active pollen formed a layer over the sucrose solution, while non-viable pollen settled at the bottom. WERNICKE, HARMS, LÖRZ and THOMAS (1978) subjected the pollen of *Nicotiana tabacum* to density gradient, centrifugation and employed sucrose with 'Percoll' (MAHESHWARI, TYAGI and MALHORTA, 1980). A 40-fold enrichment in plantlet inducing microspores was obtained for the separated fraction compared to the "unfractionated" population (MAHESHWARI, TYAGI and MALHORTA, 1980).

The induction of individual pollen grains to grow androgenically from haploids proved to be a difficult process and can only be successfully applied to a limited number of species. It was successful in *Petunia hybrida*, *Lycopersicon lycopersicum*, *Brassica oleracea*, *Nicotiana tabacum* and *Datura innoxia* (PIERIK, 1993). The utility of isolated pollen cultures do in fact have a number of special advantages over anther culture (MAHESHWARI, RASHID and TYAGI, 1982):

(i) It assures *genetic pure* regenerated plantlets. With anther culture the possibility exists that plants may originate from, not only pollen grains, but also from other somatic tissue such as the tapetum or anther wall, leading to a mixed population of plants with various levels of ploidy (including levels of high ploidy) (MAHESHWARI, TYAGI and MALHORTA, 1980).

(ii) The anther wall may have deleterious effects (possible inhibitors such as ABA and other toxic substances) on pollen grain development into embryoids (MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982; PIERIK, 1993). This can be avoided by the elimination of the anther.

(iii) An overcrowding of pollen grains within the anther is inevitable and deleterious (MAHESHWARI, RASHID and TYAGI, 1982).

(iv) Medium nutrients can be absorbed directly by pollen grains as the anther no longer acts as barrier to the transport of these nutrients (PIERIK, 1993).

(v) Through pollen culture, the possibility for anther derived callus is excluded. If callus develops from a single pollen grain it is of the same genotype, whereas if developing from many grains chimeras may occur (PIERIK, 1980).

(vi) Pollen grains can be plated and manipulated like microorganisms, making pollen culture very advantageous for mutagenesis and genetic transformation studies (PIERIK, 1993).

In certain lines of rye, unreduced gametes are produced and the ordinary culture of isolated pollen grains is in this case not so acceptable. According to MAHESHWARI, TYAGI and MALHORTA (1980) it was suggested by WENZEL (1980) to separate these larger, unreduced heterozygous pollen before initiating normal pollen culture.

In spite of all the mentioned advantages of pollen culture, it remains a difficult process, requiring a complicated nutrient medium, while the frequency of produced haploids is low.

2.2 Anther Culture

In spite of all the mentioned theoretical advantages of pollen culture, techniques for the production of microspore derived embryoids and plants mainly rely on the culture of complete anther units ("anther culture"). According to DUNWELL (1985) the successful culturing of most plant species requires for microspores to remain within the anther, after excision from the plant, for a period of time. MORRISON and EVANS (1988) also proclaimed anther culture as the method of choice for recovering haploid plants. They stated that the success of anther culture depends upon the fact that microspores are cultivated *in vitro* while it is still nourished and protected *in vivo* within the anthers until maturity.

Anther culture comprises the aseptic dissection of whole anthers from surface-sterilized flower buds and their plating on appropriate nutrient

medium. Anthers can either be plated on an agar-solified medium (where orientation is a determining factor); floated on the surface of liquid medium; supported on filter-paper bridges over liquid medium; or floated on the liquid part of double layered medium (SUNDERLAND, 1974; MAHESHWARI, TYAGI and MALHORTA, 1980; DUNWELL, 1985). Where dissection of flower buds is difficult due to small size, an intact whorl of stamens (tobacco) or a whole spike (barley) may be inoculated (MAHESHWARI, TYAGI and MALHORTA, 1980). SUNDERLAND (1974) reported that in *Nicotiana tabacum* the presence of other parts than anthers in the culture does not seem to affect pollen response provided that anthers are in direct contact with the medium. The pollen will not grow if the filament lifts the anther clear of the medium (SUNDERLAND, 1974). It was subsequently concluded that the growth-stimulus cannot be transmitted via the filament. Great care should also be taken during anther dissection as injury to anthers stimulates callus growth from an injured surface (MAHESHWARI, TYAGI and MALHORTA, 1980).

The original technique of GUHA and MAHESHWARI was developed for *Datura innoxia* and anthers were cultured on agar-solified NITSCH (1951) and WHITE (1963) media, along with hormones (GUHA and MAHESHWARI, 1964,1966). Despite success with as much as 171 species, many species proved to be recalcitrant. *Solanum tuberosum* is an important exception. DUNWELL and SUNDERLAND (1973) reported the production of haploid potatoes by means of anther culture, but the response was poor and regenerated plants proved to be dihaploids. FOROUGHI-WEHR, WILSON, MIX and GAUL (1977) used dihaploid donor plants in anther culture. Pollen was induced to form callus, differentiate and produce monohaploid plants. Response was, however, restricted to one genotype and only a few plants were obtained. The general conclusion made by MILLER and LIPSCHUTZ (1984) was that potato does not respond well to anther culture and that response was restricted to only a few genotypes.

Much effort was consequently devoted to elicit responses from recalcitrant species and also to enhance responses in those species amenable to anther culture. WERNICKE and KOHLENBACH (1975,1976) modified the original technique of GUHA and MAHESHWARI by culturing anthers in liquid medium. This resulted in higher yields of microspore derived embryoids (MAHESHWARI, RASHID and TYAGI, 1982). Further refinements to the original technique was done by SUNDERLAND and ROBERTS (1977). They cultured *Nicotiana* anthers in liquid medium and transferred the anthers to fresh medium at regular intervals (MAHESHWARI, RASHID and TYAGI, 1982). Anthers dehisched spontaneously, shedding their pollen into the liquid medium. The pollen, freed from the anther, developed into embryoids (MAHESHWARI, RASHID and TYAGI, 1982).

3. Factors Affecting Androgenesis

The main objective of anther culture is the selective stimulation of pollen to divide and form either embryoids or callus (MAHESHWARI, TYAGI and MALHORTA, 1980). Various cultural conditions and genetic factors, either single or in combination, affect the mode of microspore development from a gametophytic to a sporophytic phase. To maximize results, the identification and experimental manipulation of these factors are of great importance.

3.1 Genotype of donor plant

According to VASIL, AHUJA and VASIL (1979) increasing evidence shows that the appearance of haploids in nature and the ability of microspores to develop *in vitro* into haploid plantlets or callus is under genetic control. This was confirmed by DUNWELL (1985) who reported the production of microspore-derived plants in more than 200 species from three families, the *Solanaceae*, *Graminaceae* and *Cruciferae*. Within each species, each genotype has a characteristic response. Not only species within a genus, but even cultivars of the same species often

showed remarkable dissimilar responses in anther culture. NITSCH (1969), for example, obtained anther response in only 5 of the 12 *Nicotiana* species used in his study (MAHESHWARI, TYAGI and MALHORTA, 1980). In *Arabidopsis thaliana* (1972a) and *Lycopersicum esculentum* (1972b) GRESSHOFF and DOY found 3 out of 18 lines and 3 out of 43 lines, respectively, responsive in anther culture (MAHESHWARI, TYAGI and MALHORTA, 1980). Of the 46 *Solanum* species used (118 clones and 9 interspecific hybrids) only 19 species and 4 interspecific hybrids produced microspore-derived plantlets (IRIKURA, 1975; SOPORY and BAJAJ, 1987). Monohaploid plants were obtained in *S. verrucosum*, *S. bulbocastanum*, *S. phureja*, *S. stenotomum* and two interspecific hybrids (SOPORY and BAJAJ, 1987). Genotypic effects were also evident in rye (WENZEL, HOFFMANN and THOMAS, 1977). This phenomenon provided good evidence for strict genetic control of events during the culture phase (DUNWELL, 1985). The culture phase can be divided into two sections, namely the initial induction of divisions within the microspores and secondly the differentiation from resulting calli or embryoids (DUNWELL, 1985). Consequently, each stage is under separate genetic control. It was predicted that it would eventually be possible to determine these control mechanisms and to distinguish the genotypes which produce embryoids from those producing calli (DUNWELL, 1985). It is also generally assumed that embryoids are normally produced in the *Solanaceae* and *Cruciferae*, while the mode of development in the *Graminaceae* is via a callus phase (DUNWELL, 1985). Due to this prominent effect of genotype on plantlet formation in anther culture, it proved essential to use a wide selection of genotypes of any given species in initial experiments aimed at the production of androgenetic plants (VASIL, AHUJA and VASIL, 1979). Genotype effects on callusing, rooting and shoot formation were reported in primary dihaploids and in anther cultures of the progeny of crosses between dihaploids (FOROUGHI-WEHR, WILSON, MIX and GAUL, 1977; SIMON and PELOQUIN, 1977). Genotype also affect the origin of callus. Callus was obtained

either from pollen grains or from the anther wall (SOPORY and BAJAJ, 1987). It can either originate directly from the anther wall or from the base of the anther.

JACOBSEN and SOPORY (1978) classified 41 interdihaploids into five categories. This classification was based on response in anther culture. They attempted to improve response by conducting a number of crosses between responsive clones. A cross between $H_2$236 and $H_2$439 resulted in some positive responding progeny and of ten H_3 clones obtained, four showed no response. Pollen divisions and embryoid formation was observed in others. In clone $H_3$703 anthers produced many monohaploid embryoids which were grown into complete plants (SOPORY and BAJAJ, 1987). This, according to SOPORY and BAJAJ (1987), is "a clear case of attempting to recombine 'poor responding' genotypes to obtain and select for 'highly responding' clones, thus suggesting that genes - which positively influence the success rate - can be accumulated and selected".

Caution should, however, be taken in ascribing failure in haploid induction in recalcitrant species to genotype-effect (MAHESHWARI, TYAGI and MALHORTA, 1980). This became evident when several species and cultivars, which were previously found to be recalcitrant, proved amenable to haploid induction after being subjected to further manipulation of specific physiological conditions (MAHESHWARI, TYAGI and MALHORTA, 1980).

3.2 Physiological state of donor plant

Seasonal variations, pretreatments and age affect the physiological status of the donor plant and are important factors in determining the frequency of haploid induction (MAHESHWARI, RASHID and TYAGI, 1982).

3.2.1 Seasonal Variations

In experiments conducted by several researchers, both light and temperature affected anther response in *Solanum tuberosum, Triticum aestivum, Datura innoxia, Nicotiana knightiana* and *Brassica napus* (MAHESHWARI, TYAGI and MALHORTA, 1980; DUNWELL, 1985). *Datura innoxia* and *Nicotiana knightiana* plants showed an increase in anther response when grown at higher temperatures (20-30°C), whereas optimum results in anther response was observed when *Brassica napus* cv. 'Tower' was grown at lower temperatures (KELLER and STRINGAM, 1978; MASHESHWARI, TYAGI and MALHORTA, 1980). Experiments conducted by DUNWELL (1976) proved that photoperiod (another seasonal variable) affect anther response. *Nicotiana tabacum* cv. 'White Burley' showed a 21% increase in anther response when plants were grown under a shorter photoperiod (8h regime in stead of 16h) (MAHESHWARI, TYAGI and MALHORTA, 1980). A light intensity of 14 Klux was maintained in both cases. According to LAZAR, SCHAEFFER and BAENZIGER (1984) evidence exists for an interaction between these variables and the genotype of material used.

3.2.2 Physical Pretreatments

Certain physical pretreatments to the donor plant improved anther response, e.g. the removal of the apical region of the inflorescence; the application of hormones (*Oryza sativa)*; regular removal of older flower buds; nitrogen starvation; clipping plants at the ground level and allowing them to stand in water for one or two days (*Hordeum vulgare*); and the application of mineral nutrients (MAHESHWARI, RASHID and TYAGI, 1982). PICARD (1973) (as qouted by MAHESHWARI, TYAGI and MALHORTA, 1980) demonstrated that the removal of the apical region of the inflorescence resulted in a higher number of microspores having identical nuclei. This was correlated with enhanced embryoid formation in culture (MAHESHWARI, TYAGI and MALHORTA, 1980).

3.2.3 Hormone Treatments of Donor Plants

The effect of hormonal treatment on anther response of donor plants was illustrated in several experiments conducted. For instance, the application of an ethylene releasing compound, 2- chloroethylphosphonic acid, led to an increase in anther response when applied to *Oryza sativa* inflorescence for 48 h at 10°C (WANG, SUN and CHU, 1974). This was also demonstrated by BENNETT and HUGHES (1972) who applied this substance to wheat plants in the field (MAHESHWARI, TYAGI and MALHORTA, 1980).

3.2.4 Plant Age

MAHESHWARI, TYAGI and MALHORTA (1980) indicated a significant correlation between plant age and anther response. Evidence also exists that the frequency of androgenesis is higher in anthers harvested at the beginning of the flowering period and declines with plant age in *Datura metel*, *Nicotiana tabacum* and *Atropa belladonna* (MAHESHWARI, TYAGI and MALHORTA, 1980; DUNWELL, 1985). It is uncertain if this variable also affects the type of structure produced during culture. This reduction in response was ascribed to the deterioration in the general condition of the plants; particularly during seed set. However, in *Nicotiana tabacum* cv. 'Havanna' no difference was observed with respect to androgenesis in the "first" flowers and that of flowers developed at later stages (MAHESHWARI, TYAGI and MALHORTA, 1980).

3.3 Pollen Stage

Another critical factor affecting androgenesis is the specific developmental stage of the pollen contained in cultured anthers at the time of incubation. The reason for this is probably due to the fact that the pollen grains can be switched towards the sporophytic mode within only a narrow period of their development (MAHESHWARI, RASHID and TYAGI, 1982). For the majority of species, including potato, this critical period proved to be when the anther is in the uninucleate stage of

microspore development (midway between release from the tetrad and the first pollen grain mitosis) or at the verge of mitosis (SUNDERLAND, 1974; MASHEHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982; SOPORY and BAJAJ, 1987; PIERIK, 1993).

As this proved such a determining factor several attempts were made to correlate flower bud length and flower bud colour to this uninucleate developmental stage. FOROUGHI-WEHR, WILSON, MIX and GAUL (1977), for example, cultured light-green potato anthers with a length of 1.5 - 3.0 mm and obtained microspore-derived callus. WENZEL and FOROUGHI-WEHR (1984) cultured potato anthers 3-4 mm in length with a corresponding bud size of 4-6 mm when microspores were in the late uninucleate stage and free from starch. They found that bud size was dependent on their relative position in the inflorescence. Early flowers contained small anthers in larger buds, while the late buds were small and possessed relatively large anthers. Bud size also varied in relation to the genotype. For tobacco, flower buds are selected with petals just visible (PIERIK, 1993). Correlation between microspore developmental stage and morphological characteristics can be confusing, as a specific morphological characteristic does not always corresponds with the first microspore division (PIERIK, 1993). It should be borne in mind that the development of all the microspores within any one anther is not synchronized. Those microspores in the centre of the anther are more advanced in their development than those at the periphery. Furthermore, the exact developmental stage was found to vary between species and even between cultivars (SUNDERLAND, 1974; DUNWELL, 1985). It may also be influenced by the culture conditions and the environment under which the donor plants are grown. Consequently it proved essential that cultured anthers should occasionally be stained with acetocarmine to confirm their exact developmental stage. Acetocarmine is a general useful stain for this purpose. SUNDERLAND (1974) suggested, however, that the pollen of species possessing a low

DNA content should first be stained with Feulgen reagent for at least 2 h after fixation.

Several explanations have been offered as to why the uninucleate stage resulted (in general) in the highest anther response. MAHESHWARI, RASHID and TYAGI (1982) concluded that it could be because the pollen grains are then " in a sense uncommitted to either the androgenic or their normal mode of development". According to SOPORY and BAJAJ (1987) it could be due to the fact that the pollen did not yet reach the last stage of differentiation, i.e. the binucleate stage with starch, and is in the process of 'division' to achieve that stage. Thus being metabolically active. They also stated that once cultured and given the necessary physical or chemical stimulus, the capacity to divide may be shifted from differentiation into generative and vegetative cells to uncommitted cells. The latter continue to divide and differentiate into embryoids. SUNDERLAND (1974) explained that the highest response was observed at the stage when the vegetative cell was in the process of rapid cytoplasmic synthesis, after which response declined. If this specific stage had already been passed at the time of anther inoculation, embryogenesis could not take place. If this assumption is true, then responses obtained in older anthers must be due to grains retarded in their development and which had not yet reached this critical point at inoculation (SUNDERLAND, 1974).

It was also indicated by NITSCH, NITSCH and HAMON (1968) (as qouted by SUNDERLAND, 1974) that plantlets cannot develop from pollen after starch has been formed (the late uninucleate stage is free from starch). SUNDERLAND (1974) alternatively suggested that the hormone balance in the anther tissue becomes unfavourable for growth as the anther matures, or that another component essential to growth, is depleted.

It is, however, not always the uninucleate stage of microspore development that leads to the highest response in anther culture. For instance, in *Nicotiana tabacum* var. 'Badischer Burley' the early binucleate stage proved best, whereas in *Nicotiana tabacum cv.* 'White Burley' pollen in both the mitotic and early binucleate stages were most reactive (MAHESHWARI, TYAGI and MALHORTA, 1980).

The stage of microspore development at the time of inoculation also affects the determination of the ploidy level of regenerated plantlets. According to SUNDERLAND and DUNWELL (1977) embryoids and plantlets of higher ploidy are also produced along with haploids. In *Datura innoxia*, *Nicotiana tabacum* and *Hyoscyamus niger*, microspores at the uninucleate stage produced mainly haploids, while plantlets of higher ploidy level were developed from microspores in a more advanced developmental stage. The general conclusion made by several authors was that the more advanced the stage of microspore development of selected anthers, the higher the ploidy level of resulting embryoids and plantlets (SUNDERLAND, 1974; MAHESHWARI, TYAGI and MALHORTA, 1980; MAHEHESHWARI, RASHID and TYAGI, 1982).

3.4 Pretreatments

Various temperature treatments have been applied on inflorescences, flower buds and isolated anthers either prior to or after inoculation of several species to determine its effect on anther response. The effect of both low and high temperatures on anther response were investigated (DUNWELL, 1985). It was, however, cold temperature pretreatment of flower buds prior to inoculation that resulted in the highest increase in response. DUNWELL (1985) stated that the application of such thermal shocks do not alter the type of development undergone by cultivated microspores but only increase the number of diverted pollen grains.

3.4.1 Cold Treatments

According to MAHESHWARI, TYAGI and MALHORTA (1980) the initial and obligatory success of cold pretreatment was reported by NITSCH and NORREEL (1973) and was confirmed by several researchers. MALHORTA and MAHESHWARI (1977) and TYAGI, RASHID and MAHESHWARI (1979) applied cold treatment to flower buds of *Petunia hybrida* and *Datura innoxia*, respectively, prior to inoculation. In both experiments an increase in anther response was observed. They also found cold treatment obligatory for proper response in *ab initio* pollen cultures (MAHESHWARI, RASHID and TYAGI, 1982). Alternatively, cold treatment was applied on isolated anthers after a culture phase on nutrient medium by DUNCAN and HEBERLE (1976) and SUNDERLAND and WILDON (1979). These authors reported an increase in anther response (MAHESHWARI, TYAGI and MALHORTA, 1980). No proof could, however, be provided that cold treatment causes symmetrical microspore division. An increase in anther response was also reported following cold pretreatments performed on wheat (LAZAR, SCHAEFFER and BAENZIGER, 1985) and pepper (MORRISON, KONING and EVANS, 1986). In contrast to the findings of HAUNG and SUNDERLAND (1982), namely that cold pretreatment of 28 days at 4°C was required, KOHLER and WENZEL (1985) showed that no pretreatments were essential for improved response in barley anther cultures (MORRISON and EVANS, 1988). In *Zea mays* a treatment of the tassels at 8°C for 14 days prior to the isolation and culture of microspores was found optimum (AFELE, KANNENBERG, KEATS, SOHOTA and SWANSON, 1992). In most Solanaceae, including *S. tuberosum* ssp. *tuberosum*, a cold treatment of excised flower buds of two to three days at 6°C proved best (WENZEL and UHRIG, 1981; WENZEL and FOROUGHI-WEHR, 1984; UHRIG, 1985; JOHANSSON, 1986; POWELL and UHRIG, 1987).

The optimal dose for cold treatment varies between different plant species (MAHESHWARI, RASHID and TYAGI, 1982) and can be selected

arbitrarily (MAHESHWARI, TYAGI and MALHORTA, 1980). According to NITSCH (1974) *Datura innoxia* required a treatment of 48 h at 3°C, while detailed experiments conducted by MAHESHWARI, TYAGI and MALHORTA (1980) determined 4 days at 4°C the optimal treatment for this species. NITSCH (1974) recommended a treatment of 5°C for 72 h to excised *Nicotiana tabacum* flower buds, while SUNDERLAND and ROBERTS (1977) reasoned that a 12-day treatment at 7-8°C was optimal. A pretreatment of 5 days at 15°C was reported best for *Hyoscyamus niger* (SUNDERLAND and WILDON, 1979).

Despite the proven success of increased anther response, the molecular basis of the effect of cold pretreatment remains unanswered and several assumptions are made. NITSCH (1974) explained, for instance, that cold treatment possibly increases the frequency of embryoid formation by affecting first pollen mitosis; thus increasing the number of pollen with identical nuclei (MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982). In her experiments to prove this assumption NITSCH (1974) cultured both untreated as well as cold-treated *Datura innoxia* anthers. After five days of culture, 22% microspores of the cold-treated anthers contained identical nuclei. On the contrary, only 3% microspores of the untreated anthers contained identical nuclei. Furthermore, cold-treated anthers contained 62% dead pollen grains compared to 92% dead grains obtained in untreated anthers. No dead pollen grains were observed in either fresh or cold-treated anthers before inoculation on culture medium (MAHESHWARI, TYAGI and MALHORTA, 1980). This also proved that pollen did not become inactive (dead) due to cold pretreatment. A second possible explanation for the mechanism of cold pretreatment resulted from the work of PELLETIER and HENRY (1974). They concluded that cold treatment delays the senescense of anthers, thus maintaining a higher number of viable and embryogenic pollen grains (MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982). VASIL and NITSCH (1975) interpreted the

enhancing effect of cold pretreatment as being due to a reduction in the metabolic activity within the anther, which allows the accumulation of a higher percentage of pollen grains at the required developmental stage. Furthermore, rapid senescense of somatic tissue is a known inhibitory factor to the production of haploids which explains why cold treatment increases embryoid frequency (MAHESHWARI, TYAGI and MALHORTA, 1980).

A shift in response has also been observed in anther cultures of *Hyoscyamus albus* following cold pretreatment (MAHESHWARI, TYAGI and MALHORTA, 1980). In most cases such as *Datura innoxia* and *Hyoscyamus albus* the same cytological stage of microspore development proved the best responding one whether anthers were cultured before or after pretreatment. This critical developmental stage differed, however, between untreated and pretreated anthers of *H. albus* (MAHESHWARI, TYAGI and MALHORTA, 1980). The mid-uninucleate stage of microspore development proved best for untreated anthers and the early binucleate stage for pretreated anthers. This shift is, according to SUNDERLAND and WILDON (1979), possibly due to varying contents of inhibitors during microspore development which may be differentially affected by cold treatment at different stages.

3.4.2 High Temperature Treatments

The beneficial effect of high temperature pre-incubation and pretreatment was reported by CAPPODOCIA, CHENG and LUDLUM-SIMONETTE (1984) for *Solanum chacoense* (POWELL and UHRIG, 1987). The enhancing effect of high temperatures on androgenetic response was confirmed by BATTY and DUNWELL (1986). DUMAS DE VAULX, CHAMBONNET and POCHARD (1981) (as quoted by POWELL and UHRIG, 1987) reported similar results following their work on dihaploid *S. tuberosum* cv. $H_3$703 and *Capsicum annum* (POWELL and UHRIG, 1987). Optimal anther response was obtained in both experiments (POWELL and UHRIG, 1987).

The first reported quantitative investigation into the role of pretreatment on anther culture response in potatoes was conducted by POWELL and UHRIG (1987). From results of their experiments it was concluded that response to thermal pretreatments is genetically determined. Furthermore, the segregation of gene(s) controlling this response to temperature stress treatments was observed (POWELL and UHRIG, 1987).

3.5 Physical variables

Much attention was previously devoted to the effect of chemical factors on androgenic response of cultured anthers of different species, while the effect of physical culture conditions has only recently been studied in more detail (MAHESHWARI, RASHID and TYAGI, 1982). Physical factors such as temperature, light, anther orientation, the number of anthers cultured per vessel and the culture vessel atmosphere may all affect the process of haploid induction in anther cultures of several species (MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982).

3.5.1 Light

The enhancing effect of light on post-induction growth has been reported in many plants (MAHESHWARI, TYAGI and MALHORTA, 1980). Exposure to both continuous light during anther culture and an alternating light and dark regime must be considered. They may have different effects according to the specific requirements of the different species. For example, an alternating light and dark regime proved beneficial for increased embryoid formation in *Hyoscyamus niger* (CORDUAN, 1975), *Datura innoxia* (SOPORY and MAHESHWARI, 1976) and *Nicotiana tabacum* (SUNDERLAND, 1971). On the other hand, continuous exposure to light was preferred and proved optimal for other plants. Increasing response in anther cultures of *Datura metel* was obtained when submitted to continuous light, while the opposite effect was observed in *Anemone virginiana* (MAHESHWARI, TYAGI and MALHORTA, 1980). Furthermore,

GRESSHOFF and DOY (1974) indicated that although callus induction could be initiated even in the dark in anther cultures of *Vitis vinifera*, the induction frequency could be greatly enhanced when cultures were submitted to continuous light for the initial 24 h of the culture period, before transferring them to the dark.

According to SANGWAN-NORREEL (1977) and SUNDERLAND and ROBERTS (1977) an initial dark period followed by incubation in diffuse light resulted in increased response in pollen cultures of *Datura innoxia* and *Nicotiana tabacum*. In their experiments involving six different combinations of auxin and cytokinin concentrations, FOROUGHI-WEHR, WILSON, MIX and GAUL (1977) cultured potato anthers at 23°C in the dark and transferred them to light after differentiation was observed. In contrast to this success, no response was observed by SOPORY and BAJAJ (1987) when anther cultures were submitted to the dark. These authors found a 12 h light-dark cycle to be optimal (SOPORY and BAJAJ, 1987).

Concerning the active region of the spectrum of light, red light proved to be more suitable than blue or low intensity white light or darkness for pollen cultures of *Nicotiana tabacum* (NITSCH, 1977). On the contrary SOPORY and MAHESHWARI (1976) found red light inhibitory to anther response in *Datura innoxia*. It is therefore difficult to make a generalized conclusion concerning this aspect (MAHESHWARI, RASHID and TYAGI, 1982).

The precise regulatory role of the effect of light on anther and pollen cultures remains unexplained (MAHESHWARI, TYAGI and MALHORTA, 1980). The latter is due to obvious contradictions that emerged from experiments conducted by several researchers. For example, while certain aspects proved stimulatory in specific plants the same aspects proved inhibitory in others (MAHESHWARI, TYAGI and MALHORTA, 1980).

The effect of red light in *Nicotiana tabacum* and *Datura innoxia*, as described above, is a typical example of such a contradiction. MAHESHWARI, RASHID and TYAGI (1982) concluded that the effect of light on androgenesis could be a phytochrome or blue light-mediated effect. This aspect calls for more research.

3.5.2 Temperature

According to the results from experiments conducted on *Nicotiana tabacum* (SUNDERLAND, 1971) and *Datura innoxia* (SOPORY and MAHESHWARI, 1976) continuous culture of anthers at temperatures between 25-30°C proved optimal for the induction of embryogenesis, while both an extreme increase or decrease in temperature led to a sharp decline in response. From experiments conducted on *Brassica* species, KELLER and ARMSTRONG (1978,1979) showed that more complex requirements are involved. In *Brassica campestris* and *Brassica napus* anther response was enhanced after initial submission to high temperatures of, respectively, 35°C for one to three days and 30°C during the first 14 days of culture. This was followed by incubation at 25°C for the duration of the culture period (MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982). The resulting higher yield of microspore derived plants consisted mainly of haploids (MAHESHWARI, TYAGI and MALHORTA, 1980). Of great interest was that, when anthers of the same plants were cultured at 25°C from the beginning of the culture period, no haploids were obtained (KELLER and ARMSTRONG, 1977). BIDDINGTON and ROBINSON (1990) concluded that an initial exposure to 35°C was an absolute requirement for nearly all *Brassica* anthers. In *Oryza sativa* an increase in the formation rate of albino plantlets was observed which was directly related to high temperatures, despite the latter's enhancing effect on anther response (MAHESHWARI, RASHID and TYAGI, 1982). The obligatory effect of high temperatures for microspore embryogenesis was also demonstrated in *Capsicum* where cultures were exposed to short days (one to two days)

at 35°C (DUNWELL, 1985).

Although the actual explanation for the enhancing effect of high temperatures remains unanswered, it was predicted that it could be connected with the high temperature inhibition of protein synthesis that occur in some germinating pollen grains (DUNWELL, 1985). Exposure of cultured anthers to short periods at 35°C could inhibit proteins concerned with gametophyte synthesis which will lead to sporophyte induction. This could be due to the involvement of heat shock proteins (DUNWELL, 1985).

3.5.3 Anther Orientation

Anthers cultured in liquid medium, float on the surface in a uncontrollable manner (DUNWELL, 1985). Anther orientation can, however, be controlled and manipulated in agar solidified cultures and its effect has been studied by several researchers (SOPORY and MAHESHWARI, 1976; DUNWELL, 1985; HUNTER, 1985; SHANNON, NICHOLSON, DUNWELL and DAVIES, 1985; POWELL, BORRINO and GOODALL, 1987).

Anthers from *Datura* and *Nicotiana*, when placed on the surface of the culture medium and without submersion into the medium, produced the highest yield of microspore derived embryoids (DUNWELL, 1985). These results have been confirmed by SHANNON, NICHOLSON, DUNWELL and DAVIES (1985) in barley anther cultures. It was also found that barley anthers cultured with only one lobe in contact with the medium (i.e. on the edge or the so-called "up" position) produced higher yields of microspore derived embryoids in comparison to anthers with both locules in contact with the medium (SHANNON, NICHOLSON, DUNWELL and DAVIES, 1985; HUNTER, 1985). This orientation also led to an increase in the percentage of responding anthers (HUNTER, 1985; SHANNON, NICHOLSON, DUNWELL and DAVIES, 1985; POWELL, BORRINO and GOODALL, 1987). With this lateral or "up" orientation, the microspores in

the lower half (and in contact with the medium) underwent either none or only a limited number of embryogenic divisions which then stopped growth (DUNWELL, 1985; HUNTER, 1985). Microspores in the upper half, however, continued to develop into embryoids or callus. When barley anthers were cultured in a "flat" orientation, relative to the culture medium, microspores were generally shed onto the culture medium and no further development was observed (HUNTER, 1985). The results of SHANNON, NICHOLSON, DUNWELL and DAVIES (1985) and HUNTER (1985) were in contrast to the findings of POWELL, BORRINO and GOODALL (1987). POWELL and co-workers also cultured barley anthers in the "up" and "flat" orientations, but found that orientation did not significantly affect the percentage of responding anthers. They did, however, find a significant increase in the number of green plantlets produced when anthers were cultured in the "up" orientation compared to when they were cultured in the "flat" orientation. The majority of these plants possessed the normal barley karyotype (POWELL, BORRINO and GOODALL, 1987).

SHANNON, NICHOLSON, DUNWELL and DAVIES (1985) as well as POWELL, BORRINO and GOODALL (1987) reported that the difference in response between the embryogenic development in the upper and lower lobe (with the latter in contact with the medium) decreased when the culture medium contained 1.0 mg l^{-1} indole-3-acetic acid (IAA) and 1.0 mg l^{-1} benzyladenine (BA) in stead of 1.75 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.25 mg l^{-1} kinetin. They concluded that 2,4-D had an inhibitory effect on continued embryoid development (DUNWELL, 1985). Furthermore, the importance of temporal changes in concentrations of media components was underlined by the fact that the upper half of the anther was not in contact with the medium during the first period of culture until diffusion transferred the medium to these cells (DUNWELL, 1985). It was also indicated that structures developing from the upper half of anthers cultured on their edges, mostly resembled embryoids

rather than callus. When anthers were cultured in the "flat" orientation, the contrary proved to be true (DUNWELL, 1985).

Anther orientation proved an important factor in determining the efficiency of plantlet production from cultured anthers. The preferred orientation leading to maximum response varies, however, between different species (HUNTER, 1985).

3.5.4 Culture Atmosphere

Different volumes of culture atmosphere affected the yield of microspore derived embryoids (DUNWELL, 1985). Although little is known about the influence of the gaseous phase, it was reported that ethylene production and certain oxygen tensions could lead to either an increase or decrease in anther response.

BIDDINGTON and ROBINSON (1990) reported the production of ethylene by isolated anthers and its accumulation in culture vessels of *Brassica oleracea* var *gemmifera*. The effect of ethylene on anther cultures is, however, rare and the type of effect is genotype dependent (BIDDINGTON and ROBINSON, 1990). Ethylene production proved essential in microspore embryogenesis of *Datura metel* and *Solanum carolinense* (BIDDINGTON and ROBINSON, 1990). HORNER, MCCOMB, MCCOMB and STREET (1977) reported that the removal of produced ethylene from the culture vessel atmosphere of *Nicotiana* cultures had no affect on embryogenesis. DUNWELL (1979) found that, depending on the size of the vessel and the age of the anthers, removal of produced ethylene during the culture of *Nicotiana tabacum* could either enhance or inhibit embryo induction, embryoid survival and plantlet yield (BIDDINGTON and ROBINSON, 1990).

Furthermore, the application of silver nitrate ($AgNO_3$), an inhibitor of ethylene action in plants, led to a slight increase in embryoid induction

(BIDDINGTON, SUTHERLAND and ROBINSON, 1988; BIDDINGTON and ROBINSON, 1990). It was also reported that reduced oxygen tensions enhanced embryogenesis (DUNWELL, 1985). This was accomplished in anther cultures of, respectively, *Nicotiana tabacum* (IMAMURA and HARADA, 1981 - as quoted by DUNWELL, 1985) and *Clematis*, *Papaver*, *Anemone* (JOHANSSON and ERIKSSON, 1984 - as quoted by DUNWELL, 1985) by (i) treating anthers with nitrogen containing 2.5% or 5.0% oxygen ; and (ii) the application of CO_2 to anther cultures. This enhancing effect was explained as being due to the maintenance of the anaerobic condition as it occurs within the anther which would allow better microspore viability during the early phase of culture (DUNWELL, 1985). DUNWELL (1985) suggested that, due to the fact that no embryogenic development occurred in very small culture volumes, embryogenesis required specific oxygen tensions.

3.5.5 Anther Wall

The important and determining role of the anther wall on *in vitro* embryoid production was illustrated by the ease with which pollen contained within anthers developed into embryoids, compared to the poor response of isolated pollen (MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982). Both beneficial and inhibitory effects of the anther wall on androgenesis have been reported depending on the circumstances involved (MAHESHWARI, RASHID and TYAGI, 1982). The identification of these stimulatory and inhibitory factors requires more research (MAHESHWARI, TYAGI and MALHORTA, 1980).

NITSCH and NORREEL (1973 - as quoted by MAHESHWARI, TYAGI and MALHORTA, 1980) provided evidence for the beneficial effect of the anther wall on androgenesis. They found that a boiled water extract of cultured anthers of *Datura innoxia* promoted androgenesis of isolated pollen. This stimulatory effect has been assigned to increasing levels of certain amino acids. When glutamine and serine were added to the culture

medium, the frequency of microspore derived embryoid production increased (NITSCH, 1974a - as quoted by MAHESHWARI, TYAGI and MALHORTA, 1980). HORNER and PRATT (1979) confirmed this by analyzing the amino acid components present in *Nicotiana tabacum* anthers. It became evident that glutamine levels increased four to five-fold in cultured anthers compared to fresh, non-cultured anthers. Experiments with liquid anther cultures by SUNDERLAND and ROBERTS (1977) suggested that anther tissues conditioned the medium for growth of pollen embryoids. UHRIG (1985) confirmed this positive effect of liquid cultures. He pointed out that in liquid cultures, microspores were subjected to reduced competition for medium-nutrients. This consequently led to more available space for the individual development of embryoids. UHRIG (1985) demonstrated that the anther wall appeared open, which allowed the diffusion of developing micospores into the surrounding liquid medium. It also became evident from this work that, even under liquid culture conditions initial microspore divisions within the surroundings of the anther, were more beneficial since isolated microspores, cultured in liquid medium, preferentially developed into calli (UHRIG, 1985).

According to VASIL, AHUJA and VASIL (1979) anthers which retained their original colour and appeared healthy throughout the culture phase, showed little or no androgenic development, while mainly those that turned brown gave rise to androgenic callus or plantlets. According to them, this could be due either to the release of new substances as a result of the degeneration of anther wall layers, or the elimination of those factors which ensure normal development of microspores in nature. Microspores could now freely follow an altered developmental course, controlled only by regulated culture conditions.

Evidence also exists regarding the release of factors inhibitory to anther response from the anther wall (HEBERLE and REINERT, 1977; TYAGI,

RASHID and MAHESHWARI, 1979). For instance, when anthers of *Datura innoxia* were transferred serially to fresh medium, a two- to three-fold increase in the frequency of microspore derived embryoid formation was observed, compared to anthers cultured continuously without transfer (MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982). By transferring anthers regularly to fresh medium the inihibiting substances are diluted, allowing successive fractions of pollen grains to readily develop into embryoids (MAHESHWARI, TYAGI and MALHORTA, 1980). Contrary to the reports of VASIL, AHUJA and VASIL (1979) as discussed previously, PELLETIER and ILAMI (1972) (as quoted by MAHESHWARI, RASHID and TYAGI, 1982) and MII (1980) (as quoted by MAHESHWARI, RASHID and TYAGI, 1982) reported the inhibition of plantlet regeneration from cultured anthers as a result of rapidly senescing anthers. It was illustrated that the rapid senescence of the anther, as indicated by its browning, inhibits anther response. Senescing anthers not only fail to produce embryoids themselves but also retard the growth of potentially embryogenic pollen grains form responding anthers in serial cultures of *Datura innoxia* (TYAGI, RASHID and MAHESHWARI, 1979). This was demonstrated by an accentuated response following the removal of senescing anthers in the first transfer-action during serial cultures.

It was reported that both stimulatory and inhibitory factors could occur during the course of the same culture phase (MAHESHWARI, TYAGI and MALHORTA, 1980). For instance, during the initial culture period, stimulatory factors may be produced which induce androgenic microspore development. This was confirmed by the fact that pollen, isolated from pre-cultured anthers, gave a higher response compared to *ab initio* cultured pollen (MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982). On the other hand, the senescing anther wall may release factors inhibiting anther response, as discussed previously (PELLETIER and ILAMI, 1972 - as quoted by

MAHESHWARI, RASHID and TYAGI, 1982; HEBERLE and REINERT, 1977; TYAGI, RASHID and MAHESHWARI, 1979) during the later culture phase. The early detection and correct manipulation of these factors to enhance anther response could be of great advantage to optimize androgenesis.

3.6 Culture Medium

The development of the first basic nutrient media resulted from the work of PHILIP WHITE of the United States and ROGER GAUTHERET of France early in 1930 (VASIL, AHUJA and VASIL,1979). These original formulae, as devised for the culture of somatic tissues and which was routinely used in most of the early work on tissue cultures, have been modified in various ways to provide for the specific requirements of different species (SUNDERLAND, 1974). The three main types of basal media (Table 4) derived from the basic formulae are NITSCH's medium (1951); WHITE's (1963) general plant tissue culture medium and MURASHIGE and SKOOG's medium (MS) (1962). VASIL, AHUJA and VASIL (1979) considered the latter as the first major completely chemically defined nutrient medium. MURASHIGE and SKOOG (1962) found that inorganic, rather than organic constituents in an aqueous tobacco leave extract, led to an increase in culture growth when added to a modified White's medium. This MS medium (1962) was devised for the culture of somatic tobacco tissues (SUNDERLAND, 1974).

Table 4 **Formulations and applications of the three major types of basal media used in tissue cultures of various species** ***(SUNDERLAND, 1974; DODDS and ROBERTS, 1985)***

COMPOSITION	NITSCH (1951)	MS (1962)	White (1963)
$(NH_4)NO_3$	-	1.650 mg l^{-1}	-
KNO_3	125 mg l^{-1}	1.900 mg l^{-1}	80 mg l^{-1}
$Ca(NO_3).4H_2O$	500 mg l^{-1}	-	288 mg l^{-1}
$CaCl_2.2H_2O$	-	440 mg l^{-1}	-
$MgSO_4.7H_2O$	125 mg l^{-1}	370 mg l^{-1}	737 mg l^{-1}
$Na_2SO_4.10H_2O$	-	-	460 mg l^{-1}
KH_2PO_4	125 mg l^{-1}	170 mg l^{-1}	-
$NaH_2PO_4.H_2O$	-	-	19 mg l^{-1}
KCl	-	-	65 mg l^{-1}
$FeSO_4.7H_2O$	-	27.8 mg l^{-1}	27.8 mg l^{-1}[a]
Na_2EDTA	-	37.5 mg l^{-1}	2.67 mg l^{-1}
$MnSO_4.4H_2O$	3.0 mg l^{-1}	22.3 mg l^{-1}	1.5 mg l^{-1}
$MnSO_4.H_2O$	-	-	0.75 mg l^{-1}
$ZnSO_4.7H_2O$	0.5 mg l^{-1}	8.6 mg l^{-1}	-
H_3BO_3	0.5 mg l^{-1}	6.2 mg l^{-1}	-
KI	-	0.83 mg l^{-1}	-
MoO_3	-	-	0.0001 mg l^{-1}
$Na_2MoO_4.2H_2O$	0.025 mg l^{-1}	0.25 mg l^{-1}	-
$CuSO_4.5H_2O$	0.025 mg l^{-1}	0.025 mg l^{-1}	0.001 mg l^{-1}
$CoCl_2.6H_2O$	-	0.025 mg l^{-1}	-
myo-Inositol	-	100 mg l^{-1}	-
nicotinic acid	5.0 mg l^{-1}	0.5 mg l^{-1}	0.5 mg l^{-1}
pyridoxin.HCl	0.5 mg l^{-1}	0.5 mg l^{-1}	0.1 mg l^{-1}
thiamine.HCl	0.5 mg l^{-1}	0.1 mg l^{-1}	0.1 mg l^{-1}
glycine	2.0 mg l^{-1}	2.0 mg l^{-1}	3.0 mg l^{-1}
kinetin	-	0.40 - 10 mg l^{-1}	-
IAA	-	1 - 30 mg l^{-1}	-
Fe citrate	10.0 mg l^{-1}	-	-
sucrose	20 g l^{-1}	30 g l^{-1}	20 g l^{-1}
agar	10 g l^{-1}		
pH	6.0	5.7	5.5
APPLICATIONS:	culture of zygotic embryos (*Datura, Brassica*)	general plant tissue culture medium (*Ephedra, Ginkgo, Hordeum, Lolium, Lycopersicon, Taxus, Torreya*)	either unchanged (*Oryza, Solanum*); or in the LINSMAIER and SKOOG's (1965) modification (LS) (*Atropa, Lolium*); or in less concentrated variants of BOURGIN and NITSCH (1967-as quoted by VASIL, AHUJA and VASIL, 1979) (*Nicotiana*)

[a] White's medium requires 2.5 mg l^{-1} Fe2(SO4)3. Since ferric sulfate precipitates easily, it is more convenient to use the MS formulation in the chelated form (DODDS and ROBERTS, 1985).

The main aspect to keep in mind is that anther culture success largely depends upon the choice of basal medium (MAHESHWARI, RASHID and TYAGI, 1982). Different researchers experienced contrasting results with the different types of basal media and the importance and need to identify the influential components contained in the media; as well as to test different types of media against different plant species, became evident (SUNDERLAND, 1974; MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982). SUNDERLAND (1974) proposed that anthers of a wide range of cultivars and different genotypes should be tested wherever possible when selecting an appropriate medium. He suggested that anthers should initially be subjected to a basal medium of simple composition (e.g. NITSCH's medium, 1951). Cultures must be examined after 14-21 days to look for dividing pollen or multicellular pollen grains. If no divisions occurred but the pollen still seemed healthy and stained well; and providing that no callus have developed from somatic tissue; a more complex medium should be tested. This procedure of testing led to the observation of pollen divisions of various species of *Paeonia*, cultivars of *Freesia* and in *Capsicum annuum* and *Solanum tuberosum* (SUNDERLAND, 1974).

VII MEDIUM COMPONENTS

SUNDERLAND (1974) indicated that great care should be taken concerning the composition of culture medium when culturing Angiosperm pollen in order to prevent pollen lysis. He furthermore declared the hormonal component of the medium as critical for growth-initiation, while the composition of the basal medium (inorganic and organic substances except hormones) were less critical.

(i) Hormones (Growth Regulators)

From various reports it could be concluded that the majority of species require the addition of one or another hormone (usually an auxin and/or a cytokinin and even gibberellins) in specific concentrations (SUNDERLAND, 1974; MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982; DUNWELL, 1985; TIAINEN, 1993). SUNDERLAND (1974) predicted that the application of hormones in specific concentrations to anther cultures of different species, could trigger pollen growth alone while somatic tissues remained quiescent. Deviations from these specific hormone concentrations could induce the opposite effect, namely callus formation from somatic tissues while pollen remained quiescent (SUNDERLAND, 1974). In only a few species, *Datura innoxia* (SOPORY, 1972 - as quoted by MAHESHWARI, RASHID and TYAGI 1982), *Nicotiana tabacum* (NITSCH, 1969), *Hyoscyamus niger* (CORDUAN, 1975; RAGHAVEN, 1975) and *Saintpaulia ionantha* (HUGHES, BELL and CAPONETTI, 1975) embryoids could develop on basal medium alone without the addition of any hormones. Hormone addition to these species did, however, prove beneficial (MAHESHWARI, TYAGI and MALHORTA, 1980). NITSCH and NITSCH (1969) and NITSCH (1970 - as quoted by SUNDERLAND, 1974) classified, for instance, *Nicotiana* species as exceptional in that growth could occur in the absence of exogenous hormones. This could be due to varietal or cultural conditions (MAHESHWARI, RASHID and TYAGI, 1982). It was eventually concluded that endogenous levels in such anthers are present in a favourable balance for growth-initiation (SUNDERLAND, 1974).

According to NIIZEKI and OONO (1971) and CLAPHAM (1973) auxins are generally required for callus induction while a cytokinin together with low auxin levels proved beneficial for regeneration and plantlet formation. However, not only are the effects of auxins relative to that of cytokinins on embryogenesis better understood but there are in general little evidence concerning the role of cytokinins in microspore development

in vitro (DUNWELL, 1985; TIAINEN, 1993). Cytokinins are nevertheless routinely included in media devised for certain species such as *Solanum tuberosum* (TIAINEN, 1993). MAHESHWARI, TYAGI and MALHORTA (1980) found that cytokinins were more efficient in *Datura innoxia* and *Solanum tuberosum* while auxins gave better results in *Nicotiana tabacum* and *Lycopersicon esculentum*.

The addition of 2,4-D was recommended for anther cultures of cereal species and in *Hyoscyamus niger* this synthetic auxin promoted anther response by inducing callus growth in pollen grains which would otherwise remain non-responsive (MAHESHWARI, TYAGI and MALHORTA, 1980; DUNWELL, 1985). 2,4-D also improved callus formation in potato anther cultures, while embryoid production was inhibited (TIAINEN, 1993). It should be emphasized that auxin addition has the disadvantage of inducing disorganized development (callus formation) leading to chromosomal abberations (MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982). Direct embryoid production is therefore preferred and it is important to obtain hormonal combinations which would increase embryogenesis (MAHESHWARI, TYAGI and MALHORTA, 1980; DUNWELL, 1985).

TIAINEN (1993) reported that both the type, concentration and combination of growth regulators significantly affect embryoid and plantlet production in tetraploid potato anther cultures. The most effective growth regulators for potato anther cultures are IAA and BA (FOROUGHI-WEHR, WILSON, MIX and GAUL, 1977; SOPORY, JACOBSEN and WENZEL, 1978; TIAINEN, 1993). Concentrations of $6x10^{-6}$M IAA and $4x10^{-6}$M BA were required for optimal response in anther cultures of dihaploid potato clones (FOROUGHI-WEHR, WILSON, MIX and GAUL, 1977; SOPORY, JACOBSEN and WENZEL, 1978). TIAINEN (1993) found that when both IAA and BA were present in the medium, embryoid yield decreased compared to when the growth regulators were applied separately.

These contradictory results were ascribed to genotypic differences. WENZEL and FOROUGHI-WEHR (1984) also reported that although the addition of an auxin to the medium of *Solanum tuberosum* anther cultures was not essential, a concentration of 0.1 - 1.0 mg l^{-1} IAA is usually recommended while cytokinin addition was much more critical. A cytokinin concentration of 1 mg l^{-1} BA proved most inductive (WENZEL and FOROUGHI-WEHR, 1984; TIAINEN, 1993). Other cytokinins and auxins used in potato anther cultures by other researchers are kinetin and zeatin and 2,4-D and NAA (WENZEL and FOROUGHI-WEHR, 1984). The addition of zeatin (0.1 - 0.5 mg l^{-1}) proved essential for the initial induction in anther cultures of some recalcitrant potato genotypes (WENZEL and FOROUGHI-WEHR, 1984).

Several researchers investigated the effect of the addition of substances such as coconut milk, potato extract and crushed anthers on different species (MAHESHWARI, RASHID and TYAGI, 1982). According to MAHESHWARI, RASHID and TYAGI (1982) WEATHERHEAD and HENSHAW (1979 - as quoted by MAHESHWARI, RASHID and TYAGI, 1982) indicated that embryoid yield increased with the addition of myo-inositol, glutathione and asparagine, while NITSCH (1974) emphasized the importance of glutamine and serine.

(ii) Carbon Source

Sucrose is the most frequently used carbon source. No generalization can, however, be made to define a standard optimum concentration for different species (MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982; DUNWELL, 1985). DUNWELL (1985) made the generalization that anthers of species producing tricellular pollen *in vivo* (*Cruciferae*, *Gramineae*) required higher sucrose concentrations than anthers of species producing bicellular pollen (*Solanaceae*). For instance, in most species a 2-3% sucrose concentration gave the best results, while much higher concentrations

of 6-12% proved optimal for *Hordeum vulgare*, *Triticum aestivum*, *Brassica campestris* and *Solanum tuberosum* (MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982). According to SOPORY (1979) and KELLER, RAJHATHY and LACAPRA (1975) optimum results have been obtained when anthers of *Solanum tuberosum* and *Brassica campestris* were cultured initially on a high sucrose media and thereafter transferred to a media containing lower sucrose levels for optimal post-induction growth. This was ascribed to a possible osmoregulatory role of sucrose (SOPORY, 1979). This corresponded with the suggestion that, despite its main role as carbohydrate source, sucrose can also have, when present in higher concentrations, some osmoregulatory role at the time of induction (MAHESHWARI, TYAGI and MALHORTA, 1980). Once growth was initiated, higher concentrations however, had no further beneficial effect (MAHESHWARI, TYAGI and MALHORTA, 1980). In a study to determine the effect of maltose in comparison with sucrose as an alternative carbon source in potato anther cultures, BATTY and DUNWELL (1989) illustrated that sucrose favoured embryoid induction while maltose promoted the development of embryoids into plantlets. They concluded that this difference was due to nutritional rather than osmotic differences between the two sugars.

(iii) Minerals and Salts

According to DUNWELL (1985) the exact effect of the major salts on the different microspore development stages during the culture phase is not known. However, the effect of iron had been identified by VAGERA and JILEK (1984 - as quoted by DUNWELL, 1985) during studies on tobacco anther cultures. Embryoids could develop to the globular stage in medium containing agar and sucrose. The addition of iron was, however, essential for complete plantlet development (DUNWELL, 1985).

(iv) Activated Charcoal

The beneficial effect of activated charcoal was illustrated as anther

response was greatly enhanced by its addition to agar solidified cultures of *Nicotiana* species (NAKAMURA and ITAGAKI, 1973; ANAGNOSTAKIS, 1974; WERNICKE and KOHLENBACH, 1976), *Anemone virginiana* (JOHANSSON and ERIKSSON, 1977), *Secale cereale* (WENZEL, HOFFMANN and THOMAS, 1977), *Solanum tuberosum* (SOPORY, JACOBSEN and WENZEL, 1978) and *Datura innoxia* (TYAGI, RASHID and MAHESHWARI, 1980a). Although its exact mechanism remains unexplained, it was postulated that activated charcoal adsorbs inhibitors either originally present in the agar medium ór those released from the senescing anther wall and degrading sucrose in the medium (WERNICKE and KOHLENBACH, 1976; WEATHERHEAD, BURDON and HENSHAW, 1978). Care should, however, be taken as charcoal could also adsorb hormones and other beneficial components from the nutrient medium (WEATHERHEAD, BURDON and HENSHAW, 1978).

Although it was reported that the utilization of liquid media [section (v)] generally resulted in increased anther response compared to agar solidified media, KOHLENBACH and WERNICKE (1978) observed an inhibition in response following the application of activated charcoal to liquid cultures of *Nicotiana tabacum* and *Datura innoxia*. They concluded that agar solidified media containing charcoal gave the best response. This was confirmed by TYAGI, RASHID and MAHESHWARI (1980b).

(v) Liquid versus Agar Solidified Media

The enhancing effect of liquid media in comparison with agar solidified media on androgenesis was discovered during the seventies (MAHESHWARI, TYAGI and MALHORTA, 1980). According to WERNICKE and KOHLENBACH response in anther cultures of *Scopolia cariolica* (1975) and *Nicotiana tabacum* (1976) were enhanced when using liquid media in stead of agar solidified media. Liquid media containing activated charcoal also proved optimal in several studies conducted on *Solanum*

anther cultures (UHRIG, 1985; POWELL and UHRIG, 1987). The enhancing effect of liquid media could be due to, for instance, the removal or better diffusion of inhibitory factors present in the anther wall; less competition for and more efficient supply of nutrients and the elimination of substances present in the agar which are inhibitory to androgenesis (MAHESHWARI, TYAGI and MALHORTA, 1980). UHRIG (1985) added that developing microspores were diffused more efficiently into the surrounding medium, leaving more space available for inividual development of embryoids. Initial divisions of microspores should, however, occur within the anthers, since isolated microspores cultured in liquid culture medium develop into calli (UHRIG, 1985).

DUNWELL (1985) considered agar and agarose part of the culture medium rather than inert additions to defined media. Of the two, agarose was preferred to agar by certain researchers as gelling agent (HENRY, DE BUYSER, GUENEGOU and ORY, 1984 - as qouted by DUNWELL, 1985; LYNE, BENNET and HUNTER, 1985 - as quoted by DUNWELL, 1985), while JOHANSSON (1988) found both gelrite and agarose superior to agar for potato shoot multiplication. Some of the negative effects caused by agar includes its ability to alter the availability of cytokinin and other hormones and thus influencing the type of microspore development (DUNWELL, 1985). Agar also contains compounds deleterious to microspore embryogenesis such as iron-impurities (DUNWELL, 1985).

CALLEBERG and JOHANSSON (1993) investigated the effect of 3% potato starch in combination with gelrite as gelling agent on potato anther cultures. This resulted in a higher embryoid yield compared to medium containing only gelrite. The stimulating effect of potato starch compared to agar in tissue cultures, was due to the absence of inhibitory substances (JOHANSSON, 1983; KOHLENBACH and WERNICKE, 1978). CALLEBERG and JOHANSSON (1993) further concluded that, except for its role as gelling agent, starch may also be of nutritional and/or osmotic value.

VIII REGENERATION MEDIUM

Media for plantlet regeneration from pollen callus differ from growth-initiation media only in respect of the hormonal components (SUNDERLAND, 1974). GUHA, IYER, GUPTA and SWAMINATHAN (1970 - as quoted by SUNDERLAND, 1974)) developed a medium for *Oryza sativa* var. *indica* which allowed direct plantlet formation from pollen without initial callus formation. SUNDERLAND (1974) suggested that anthers should be transferred from the growth-inducing medium to the regeneration medium during an early culture stage, for instance shortly after growth commenced.

Embryoid regeneration medium, for the development of potato embryoids into plantlets, differ from the initiation medium in that it is supplemented with 0.1 mg l^{-1} gibberellic acid (UHRIG, 1985). The sucrose level is also reduced from 6% to 2% to prevent extensive callus formation (UHRIG, 1985). Regenerated plantlets are subsequently subcultured to test tubes containing half strength LINSMAIER and SKOOG (1965) without growth regulators (UHRIG, 1985).

IX OBJECTIVES

The Diploid Potato Breeding Program were initiated at the Vegetable and Ornamental Plant Institute at Roodeplaat, Pretoria and comprises the combination of conventional and unconventional breeding techniques. The program is based on the reduction of the ploidy level of cultivated potatoes, from the tetraploid to the dihaploid level, to facilitate crossings with diploid wild species for gene-transfer and subsequent crop improvement. Anther culture is the preferred tissue culture technique for the rapid reduction of the ploidy level. The main objective of this study was to try and determine and optimize anther culture conditions for *different potato genotypes* to assure optimal response *in vitro*. The

androgenetic ability and culture requirements of various potato genotypes were investigated and compared. The diploid wild potato species *Solanum canasense* (obtained from CIP), two dihaploid breeding lines 87.2002/3 and 87.2002/7 (obtained from the Max Plank Institute for Plant Breeding, Köln) and tetraploid cultivars Atzimba, Buffelspoort and BP_1 (from the potato breeding program) were used. The specialized tissue culture techniques used in the study comprised alternative culture methods, media manipulation and microtechniques.

CHAPTER 2

MICROTECHNIQUE

I INTRODUCTION

The potato belongs to the genus *Solanum* and have a basic chromosome number of 12. Cultivated potato, *Solanum tuberosum* ssp. *tuberosum* is essentially autotetraploid with a chromosome number 2n=4x=48. This tetraploid nature and consequent tetrasomic inheritance, as well as a limited genetic base and a high degree of heterozygosity and sterility, complicates potato breeding. Due to these barriers conventional breeding methods, propagation of the crop by means of seed and overall crop improvement is slow. To overcome these barriers a combination of conventional and unconventional breeding techniques was suggested for potato breeding schemes. The main objective is to extend the crop's limited genetic base by means of crossings with diploid wild species and to reduce the heterozygosity and complexity of the autotetraploid material (CHASE, 1963; BAJAJ and SOPORY, 1986). This breeding scheme was developed on the basis of dihaploids and aimed at tetraploids with maximal heterozygosity (CHASE, 1963). It comprises dihaploid production by means of parthenogenesis, monohaploid production from dihaploids by means of androgenesis and the development of homozygotic dihaploids and tetraploids following chromosome doubling (CHASE, 1963). The latter can be obtained either by means of somatic polyploidization, meiotic polyploidization or protoplast fusion (ROSS, 1986).

The first reduction step from the tetraploid to the dihaploid level can be accomplished either by means of parthenogenesis, which comprises interspecific crosses of tetraploids with the diploid wild pollinator

Solanum phureja (clones IVP_{35} / IVP_{101}) or by means of specialized anther culture techniques (*in vitro* androgenesis). *In vitro* androgenesis proved, however, more successful in monohaploid production from dihaploid individuals (SOPORY, JACOBSEN and WENZEL, 1978; WENZEL and UHRIG, 1981; SOPORY and BAJAJ,1987). Monohaploids are the only source for the production of large numbers of fertile, homozygous dihaploid and tetraploid clones by means of diploidization and clonal propagation in only one generation (VASIL, AHUJA and VASIL, 1979; SOPORY and BAJAJ, 1987; MORRISON and EVANS, 1988). This is a much faster alternative - compared to conventional inbreeding methods of six to eight years - for the production of homozygosity in self-pollinating species.

The main objective of *in vitro* androgenesis by means of anther culture is the formation of a sporophyte from the male gametophyte (SANGWAN and SANGWAN-NORREEL, 1987) or, in short, the selective stimulation of microspores (pollen) to divide and form embryoids (MAHESHWARI, RASHID and TYAGI, 1982). Microspores represent the beginning of the male gametophyte generation (DODDS and ROBERTS, 1985). These microspores are formed through the process of microsporogenesis. HEBERLE-BORS (1982) was the first to categorize this process into six different developmental stages following his work with *Nicotiana tabacum* anthers. The first step in this process of microsporogenesis is where the primary sporogenous cells of the young, developing anther divide mitotically - together with the development of the pollen sac wall - to produce the microspore mother cells (MMC) or microsporocytes (FAHN, 1974). HEBERLE-BORS (1982) classified this as the pre-meiotic stage, where the microspore mother cells are enlarged and contains a nucleus in the interphase stage. At first no callose can be distinguished at this stage but just before the next division the primary walls of the microspore mother cells are replaced by thick layers of callose (Figure 5A) and massive protoplasmic strands connect the cells.

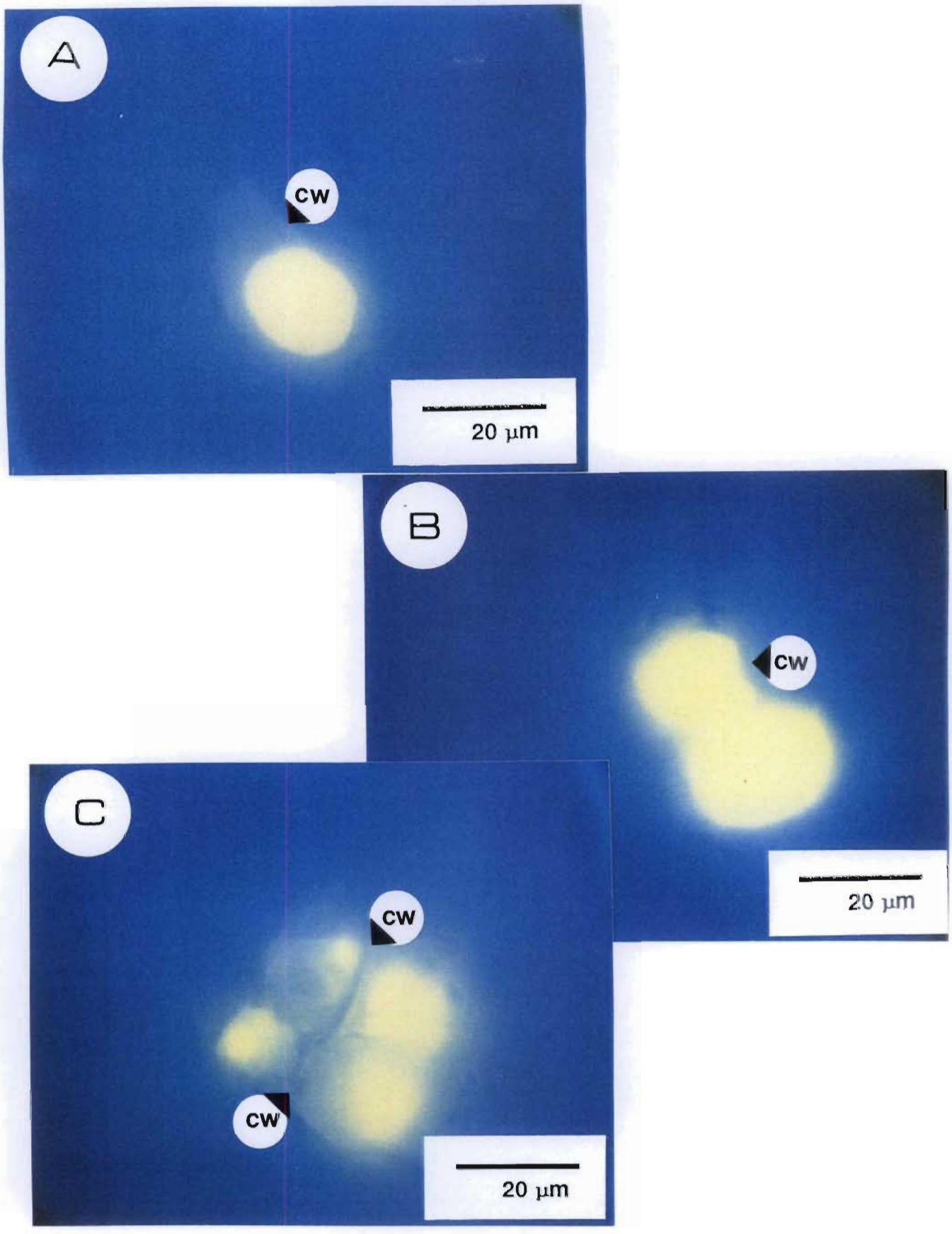


Figure 5 (A - C) The process of microspore development from the microspore mother cell, to the dyad and tetrad stages during microsporogenesis.

A. Microspore mother cell surrounded by a thick callose wall (cw).
B. A dyade of microspores surrounded by a callose wall (cw).
C. A tetrad cluster of microspores surrounded by a callose wall (cw).

The second stage is the meiotic stage where the microspore mother cells now divide meiotically to first produce a dyad (Figure 5B) and, following a second division, a tetrad of microspores (i.e. four haploid microspores) (FAHN, 1974). This is the third stage classified by HEBERLE-BORS (1982) as the tetrad stage. Each tetrad cluster (Figure 5C) is enclosed within a thick callose wall, but without plasmodesmatal connections. The microspores of each tetrad separate from one another and lie freely in the pollen sac. The young microspore contains a single central or polar situated nucleus and a large vacuole. As the microspore matures the vacuole is replaced by the enlarging nucleus and increasing amount of cytoplasm (FAHN, 1974). A mature microspore is surrounded by a thin cellulose wall, the intine, with on its outside another layer, the exine. The latter consists of a though substance, *sporopollenin*, which gives durability to the microspore. At this stage microspores are in the so-called uninucleate stage of development (Figure 5D). The proven optimal stage for maximal androgenetic response in anther cultures of various species and genotypes, including potato (FOROUGHI-WEHR and WENZEL, 1993). Microspores now undergo a mitotic division of the chromosomes, without a cytoplasmic division (karyokinesis), producing a cell with two haploid nuclei, namely the generative and the vegetative nuclei (STANSFIELD, 1983). In nature, pollen is usually shed at this stage of development (STANSFIELD, 1983). HEBERLE-BORS (1982) distinguished two stages, namely the first mitotic stage (Figure 5D), where both uninucleate and binucleate microspores can be present as microspores are still in the process of mitosis, and the binucleate stage (Figure 5E) where the process is completed and only binucleate microspores are present. During the late binucleate stage the nuclear-body (nucleolus) becomes prominent and starch deposition can occur.

In contrast with gametic cells *in vivo*, which would develop to produce the male gametophyte or sperm cell, gametic cells (microspores) contained in cultured anthers would divide symmetrically under *in vitro*

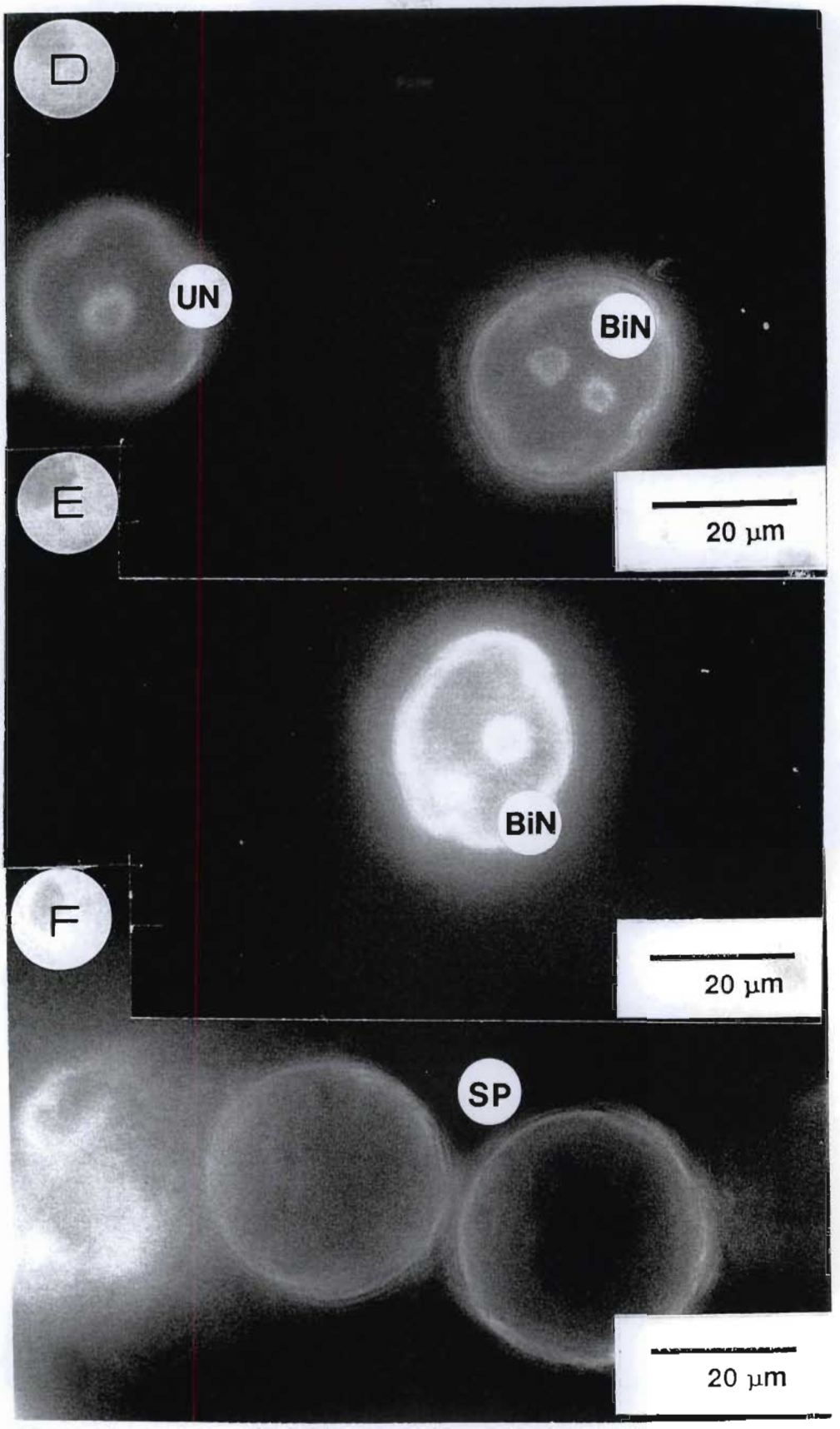


Figure 5 (D - F) The process of microspore development indicating the uninucleate and binucleate developmental stages as well as sterile microspores.

D. The uninucleate **(UN)** and binucleate **(BiN)** stages of microspore development ocurring together in the "first mitotic stage".
E. The binucleate stage of microspore development indicating the two nuclei (generative and vegetative).
F. Sterile pollen grains (microspores) **(SP)**.

conditions and eventually give rise to the sporophyte (a plant) (SANGWAN and SANGWAN-NORREEL, 1987). This switch towards the sporophytic mode can occur within only a limited period of microspore development (MAHESHWARI, RASHID and TYAGI, 1982; SANGWAN and SANGWAN-NORREEL, 1987). This period is characterized by intense metabolic activity within the anther, including RNA and DNA synthesis (VASIL, AHUJA and VASIL, 1979). The exact limits of the most favourable period for *in vitro* androgenesis depend, however, on the genus (SANGWAN and SANGWAN-NORREEL, 1987). Furthermore, various culture conditions and genetic factors, either singly or in combination, have been reported to control and affect the specific mode of microspore development from a gametophytic to a sporophytic phase (DUNWELL, 1985; SANGWAN and SANGWAN-NORREEL, 1987). These variables include the genotype and physiological state of the donor plant, pre-treatment of selected flower buds, certain physical variables, the composition of the culture medium and the stage of microspore development of cultured anthers at the beginning of the culture phase (SUNDERLAND, 1974; VASIL, AHUJA and VASIL, 1979; MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982; DUNWELL, 1985; SANGWAN and SANGWAN-NORREEL, 1987; SOPORY and BAJAJ, 1987).

This study aimed to investigate the effect of plant age and the stage of microspore development of selected anthers on the androgenetic response of different *Solanum* genotypes. Concerning plant age, the first experiment was conducted to determine whether anthers from flowers harvested at the beginning of the flowering period have greater androgenetic ability compared to those harvested later and at the end of the flowering period. This study was based on the indications from MAHESHWARI, TYAGI and MALHORTA (1980) and DUNWELL (1985) that the frequency of androgenesis decreases with plant age in anthers of certain *Datura*, *Nicotiana* and *Atropa* species. A significant correlation between plant age and anther response was also indicated by MAHESHWARI, TYAGI and MALHORTA (1980).

The second experiment aimed to determine a specific flower bud length, or length-range, most representative of the uninucleate stage of microspore development for different potato genotypes. This was determined by means of fluorescent techniques. For the majority of species, including potato, optimal androgenetic response is achieved when microspore-development of selected anthers is midway between release from the tetrad and the first pollen grain mitosis, or the so-called uninucleate stage of microspore development (SUNDERLAND, 1974; MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982; SANGWAN and SANGWAN-NORREEL, 1987; SOPORY and BAJAJ, 1987; PIERIK, 1993). According to NITSCH (1969) a correlation exists between the development of the flower bud and its anthers. It therefore proved advantageous to accurately select flowers in the correct developmental stage, merely on the grounds of flower bud size. Several attempts were therefore made to correlate a specific potato flower bud length to the uninucleate stage of microspore development. WENZEL and FOROUGHI-WEHR (1984) indicated that potato anthers, 3-4 mm in length, with a corresponding flower bud size of 4-6 mm, contained microspores in the late uninucleate stage, that are free of starch. No sporophytic development and subsequent formation of macroscopic structures can occur after the depositioning of starch (FOROUGHI-WEHR and WENZEL, 1993). It must be kept in mind, however, that due to genetic differences the correlation between flower bud size and the stage of microspore development can differ between different cultivars (KASPERBAUER and COLLINS, 1974; PIERIK, 1993) and flower bud size itself varies in relation to the genotype (PIERIK, 1993). Furthermore, the size of potato flower buds that contain anthers in the right developmental stage, depends on the relative position of the flowers in the inflorescence. Early flowers contained small anthers in relative large buds, while the late buds were small and contained larger anthers. Another aspect to keep in mind is that the development of the microspores contained in any one anther is not synchronized and microspores in the centre of the anther are more

advanced in their development than those at the periphery (PIERIK, 1993).

Thirdly, the microscopic changes taking place during the culture period in the anthers of different *Solanum* genotypes were investigated. The aim was to determine whether microspores divide during the culture phase and if microspore derived embryoids or calli were produced. The difference in response concerning these microscopic divisions (if any) between three different potato genotypes was compared. The latter was mainly done due to the high androgenetic response of dihaploid breeding line 87.2002/3 observed in all anther culture experiments, while cultivar Atzimba and the diploid wild species *Solanum canasense* showed no response.

II MATERIALS AND METHODS

1. Plant Material

The material incorporated into this study included the commercial tetraploid cultivar, Atzimba, with little to no *in vitro* androgenetic response, the diploid wild species *Solanum canasense* and the dihaploid breeding line 87.2002/3. The latter was obtained from the Max Planck Institute for Plant Breeding, Köln, and was selected for its high androgenetic response. The diploid wild species *Solanum canasense* was propagated *in vitro* from virus free seed, obtained from CIP, Peru. Tuberization occurred under glass house conditions. Virus free potato tubers of selected genotypes were grown in a glass house under controlled conditions. A light intensity of 200 µmol $m^{-2}s^{-1}$, day/night temperature of 21°C/14°C and a 16 h photoperiod proved to be essential for optimum flower formation. For the first experiment, only responsive dihaploid breeding line 87.2002/3 was utilized.

2. Procedure

Experiment 1: Determination of effect of plant age on androgenetic response in potato

Flowers from dihaploid breeding line 87.2002/3 were selected and harvested one week after the appearance of the first flowers on the parent plants. For the duration of the flowering period, flowers were harvested every seven days. All flowers received a cold pre-treatment at 6 - 9°C for two days, after which anthers were dissected aseptically and cultured on an initiation medium (3. Medium Composition) for three months. The five anthers from each flower bud were cultured together in a petri-dish and five petri-dishes were considered as one replication. Results were taken every four weeks. This experiment was repeated twice. The first flowering period (trial 1) commenced in August 1994. The first flowers of this trial appeared on 15 August 1994 and the first replication was harvested seven days later on 22 August 1994. The flowering period lasted for 42 days and six replications were harvested and cultured. The second trial commenced in October 1994 and consisted of five replications.

Experiment 2: Rapid assessment of microspore developmental stages in potato using DAPI

A range of potato flower buds, varying in length, were selected from the three genotypes Atzimba, 87.2002/3 and *S. canasense*. Flower buds of approximately the same length were roughly divided into groups (Table 5). For this study, three flowers were selected from each group and measured. From each of these three flowers, two of the five anthers contained in a flower bud, were used. Both flower bud- and anther length as well as anther colour were taken into consideration. In order to determine the different stages of microspore development, the anthers were dissected from the selected flower buds and each anther was squashed on a separate microscope slide and stained in DAPI-solution (4,6-diamidino-2-phenylindole). The microspore developmental stages

were studied with a fluorescence microscope. The number of microspore mother cells, diades, tetrades, uninucleate-, binucleate- and sterile pollen grains were taken into consideration for data analysis.

CLASSIFICATION OF FLOWER BUDS INTO DIFFERENT LENGTH GROUPS

Table 5 Classification of 87.2002/3, Atzimba and *S. canasense* flowers into four groups according to flower bud length.

LINE / CULTIVAR	GROUP	FLOWER BUD LENGTH RANGES
87.2002/3	I	3.0 - 3.9 mm
	II	4.0 - 4.9 mm
	III	5.0 - 5.9 mm
	IV	6.0 - 6.9 mm
Atzimba	I	4.0 - 4.9 mm
	II	5.0 - 5.9 mm
	III	6.0 - 6.9 mm
	IV	7.0 - 7.9 mm
S. canasense	I	3.0 - 3.9 mm
	II	4.0 - 4.9 mm
	III	5.0 - 5.9 mm

STAINING PROCEDURE WITH DAPI (VERGNE, DELVALLEE and DUMAS, 1987):

(i) Prepare a 1 mg ml^{-1} stock-solution of DAPI in distilled water.

(ii) Add 1 µL of the stock-solution to 1 ml citrate-phosphate buffer at pH 4.

(iii) Gently squash anther in a drop of this solution on a microscope slide.

(iv) Cover with cover-slip and study with fluorescence microscope after 5 - 10 minutes.

Experiment 3: Assessment of mode of microspore development and embryoid formation in cultured potato anthers by means of microtome sections.

This study, concerning cell structure within cultured anthers, was based on the procedure described by FEDER and O'BRIEN (1968). It comprised the examination of fixed and dehydrated material, embedded in a solid matrix with subsequent sectioning and staining. All material and chemicals used, had to be at room temperature before use, since condensation could occur leading to the dilution of solvents, (FEDER and O'BRIEN, 1968). Flower buds were selected from the three genotypes Atzimba, 87.2002/3 and *S. canasense* and anthers were fixed according to the experimental design described below (Figure 6).

EXPERIMENTAL DESIGN

1. A minimum of 70 flower buds were selected on one day of each genotype.
2. Ten flower buds received **NO** cold pre-treatment and 1 anther from each of these flower buds was removed and fixed (Treatment 1).
3. The remaining 60 flowers received a cold pre-treatment for 2 days.
    - 3.1 Ten of the 60 flowers were selected after two days cold treatment and one anther from each flower was fixed (Treatment 2).
    - 3.2 The remaining flower buds were dissected and 25 anthers (anthers from 5 flowers) were cultured per petri-dish on standard initiation medium (i.e. 10 petri-dishes of 25 anthers each).
4. The petri-dishes were divided in 2 groups of 5 each:
    - 4.1 From the first group, 2 anthers were removed from each of the 5 petri-dishes after 7, 14, 21, 28, 35 and 42 days respectively and fixed as described below (Treatments 3, 4, 5, 6, 7 and 8).
    - 4.2 The anthers from the second group of petri-dishes

Figure 6 Schematic outline of experimental design for anther selection and fixation for microtome sectioning

<table>
<tr><td colspan="4">Selection of 70 flowers[1]</td></tr>
<tr><td>10 Flowers
(No cold pre-treatment; 1 anther fixed per flower bud)[2]</td><td colspan="3">60 Flowers
(2 Days cold pre-treatment)[3]</td></tr>
<tr><td rowspan="8"></td><td>10 Flowers
(1 anther of each flower bud fixed)[3.1]</td><td colspan="2">50 Flowers
(25 Anthers cultured per petri-dish, 10 petri-dishes divided into two groups)[3.2]</td></tr>
<tr><td rowspan="7"></td><td>Group I (5 petri-dishes)[4.1]:</td><td>Group II (5 petri-dishes)[4.2]:</td></tr>
<tr><td>2 Anthers fixed from each petri-dish after <u>7 days</u> on medium</td><td colspan="2">Anthers cultured for 28 days - transferred to fresh medium</td></tr>
<tr><td>2 Anthers fixed from each petri-dish after <u>14 days</u> on medium</td><td>After further 14 days of culture; 2 anthers fixed from each petri-dish</td><td>After further 28 days of culture; 2 anthers fixed from each petri-dish</td></tr>
<tr><td>2 Anthers fixed from each petri-dish after <u>21 days</u> on medium</td><td colspan="2"></td></tr>
<tr><td>2 Anthers fixed from each petri-dish after <u>28 days</u> on medium</td><td colspan="2"></td></tr>
<tr><td>2 Anthers fixed from each petri-dish after <u>35 days</u> on medium</td><td colspan="2"></td></tr>
<tr><td>2 Anthers fixed from each petri-dish after <u>42 days</u> on medium</td><td colspan="2"></td></tr>
</table>

remained on the culture medium for 28 days and were then transplanted to fresh initiation medium. After further culture periods of 14 and 28 days, respectively, on the fresh medium, two anthers were removed and fixed from each of the 5 petri-dishes (Treatments 9 and 10).

(i) FIXATION:

Anthers were fixed (on the indicated days) in small specimen-tubes containing 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 6.8) for 12 h.

(ii) DEHYDRATION:

Dehydration occurred in a series of alcohols. Fixed material was transferred to 2-methoxyethanol. Two changes were made of this solvent after 12 h. Fixed anthers were then transferred to, respectively, 100% ethanol, 100% n-propanol and finally 100% n-butanol. At each step solvents were changed twice before transferring the material to the next solvent-type. Material can be stored without damage for several months in propanol or butanol at -20 to -25°C (FEDER and O'BRIEN, 1968).

(iii) EMBEDDING IN PLASTIC:

The fixed, dehydrated material was embedded in glycol methacrylate, as this plastic gave better results than wax embedding (FEDER and O'BRIEN, 1968). Material was first infiltrated at room temperature with a monomer mixture consisting of 188 ml glycol methacrylate (the monomer), 120 ml polyethylene glycol 200 (plasticizer) and 1.0 g benzoyl peroxide (catalyst). This solution was mixed on a magnetic stirrer for 1 h and filtered before use. It can be stored for several months in a refrigerator or several days at room temperature in a dark cabinet,
provided that the container is only partly filled and not tightly sealed as the exclusion of atmospheric oxygen could cause polymerization in the dark within one or two days (FEDER and O'BRIEN, 1968). Room temperatures higher than 27°C could also cause premature polymerization (FEDER and O'BRIEN, 1968). If polymerized plastic blocks

were too hard and brittle, more plasticizer had to be added to the monomer mixture, while the catalyst-concentration of blocks containing gas bubbles around the material or blocks that were too soft had to be adjusted (FEDER and O'BRIEN, 1968). Material was placed in the monomer mixture and the latter was changed twice after intervals of 24 h. Anthers were then transferred to aluminium weighing dishes, 1 cm deep and a 4.5 cm diameter, together with sufficient monomer mixture to cover the anthers. A second aluminium dish was placed on top of the first and pressed gently to ensure an airtight seal (FEDER and O'BRIEN, 1968). This was placed in an oven at 60°C for 24 h during which the mixture changed into a hard, semi-transparent plastic. Aluminium dishes were removed from the hardened plastic blocks. After the necessary trimming was done by means of a jeweller's saw and fine file, the plastic blocks containing the anthers were sectioned with a dry, sharp glass knife on a Reichert-Jung Autocut 2040 ultramicrotome. The glass knives were cut with a Leica Reicher Knifemaker. During trimming, care had to be taken to assure correct orientation for sectioning (FEDER and O'BRIEN, 1968). The thin cross-sections of 3-5 µm were placed in a drop of distilled water on a microscope slide and left to dry on a hot plate for 24 h. Eight sections were placed per slide.

(iv) *STAINING OF SECTIONS:*

Sections were stained according to the Periodic Acid Schiff's (PAS) reaction and counterstained with toluidine blue (FEDER and O'BRIEN, 1968). As material was fixed in aldehydes, an aldehyde block had to be carried out using 2-4 dinitrophenylhidrasien (DNPH). Slides were placed in 1% periodic acid for 10 minutes, and rinsed for 10 minutes in running water after which they were placed in Schiff's reagent for 30 minutes. This was followed by another rinse in running water for 5 - 10 minutes. Sections were counterstained with toluidine blue (pH 4.4) for 3 - 5 minutes and rinsed in ordinary tap water until the plastic was nearly free of the stain. Distilled water was used for the final rinse. Sections were left to dry (without heating) after which a cover slip was applied over the

mountant, entilin. According to FEDER and O'BRIEN (1968) RNA stains purple, DNA blue or blue-green, lignin and some polyphenols green, some polyphosphates, polysulfates, starch and some complex polysaccharides red using this technique.
Sections were studied by means of a Nikon light microscope and photographed.

3. Medium Composition

The initiation medium utilized in Experiment 1 and Experiment 3 consisted of the basal medium of LINSMAIER and SKOOG (1965) at half strength, supplemented with 5 g l^{-1} activated charcoal, 8 g l^{-1} agar, 60 g l^{-1} sucrose and the filter-sterilized growth regulators 0.2 mg l^{-1} thiamine HCl, 50 mg l^{-1} (myo)-inositol, 1.0 mg l^{-1} benzyladenine, 0.1 mgl^{-1} indole-3-acetic acid, 7.5 mg l^{-1} asparagine and 7.0 mg l^{-1} glutamine. The pH was adjusted to 5.8

4. Statistical Analysis

Complete randomised designs were used in Experiments 1 and 2. The number of responding anthers as well as the total number of embryoids produced were recorded. From this data the variables embryoid frequency (number of embryoids produced in 100 anthers), anther response (% anthers that form embryoids) and anther productivity (number of embryoids produced from responding anthers) were determined. Data was log-transformed and analyzed with the general linear model (GLM), using the Poisson distribution. For all three variables investigated (embryoid frequency, anther response and anther productivity), all differences reported were significant ($P<0.05$), unless otherwise indicated.

III RESULTS AND DISCUSSION

Experiment 1: Determination of effect of plant age on androgenetic response in potato

No response was observed from the second trial and data was only analyzed for Trial 1. This implies that the androgenetic ability of potato anthers decreases with a decreasing flowering period, as the second trial commenced a month later than Trial 1. Trial 1 consisted of 6 replications. No response was observed, however, for time 2 (flowers harvested 14 days after the beginning of the flowering period) and this group was consequently not included for data analysis.

Significantly higher embryoid frequencies (Table 6b and Figure 7) were obtained for both times 1 (124) and 2 (112) compared to times 3 (16) and 4 (12). No significant differences were found between times 1 and 2. From Table 7b it can be seen that in terms of anther response, both times 1 (40) and 2 (36) resulted in significantly more anthers responding compared to time 4 (4). Again, no significant difference was found between times 1 and 2. There was little difference between both the embryoid frequency response of time 1 (124) and time 2 (112) (Table 6b) and the anther response of time 1 (40) and time 2 (36) (Table 7b). After the 21 day treatment a sharp decrease in both embryoid frequency and anther response was observed (Figure 7). No significant difference was found in terms of anther productivity (Table 8b).

As embryoid yields and the number of responding anthers significantly increased from flowers harvested at days 7 and 21, it was concluded that the anthers from flowers harvested 7 days (time 1) and 21 days (time 2) after the appearance of the first flowers on the donor plants, were androgenetically more active than those harvested after 28 (time 3) and 35 days (time 4). This agrees with the results of MAHESHWARI, TYAGI and MALHORTA (1980) and DUNWELL (1985), namely that the frequency

of androgenesis decreases with plant age. Factors which could cause a variation in these results and which must be kept in mind, are the relative position of the flowers on the inflorescence and the stage of microspore development of selected anthers. For instance, flowers differing in age and development could possibly be selected during the various collection periods, as the youngest potato flowers are situated at the apex of the inflorescence. Older flowers, with anthers containing microspores at more advanced developmental stages, are situated in the centre and basal parts of the inflorescence. It would appear that the "early" flowers of responsive potato genotypes (harvested not later than three weeks after the appearance of the first flowers) give the best androgenetic response, providing that these flowers contain anthers in the uninucleate stage (WENZEL and FOROUGHI-WEHR, 1984) of microspore development.

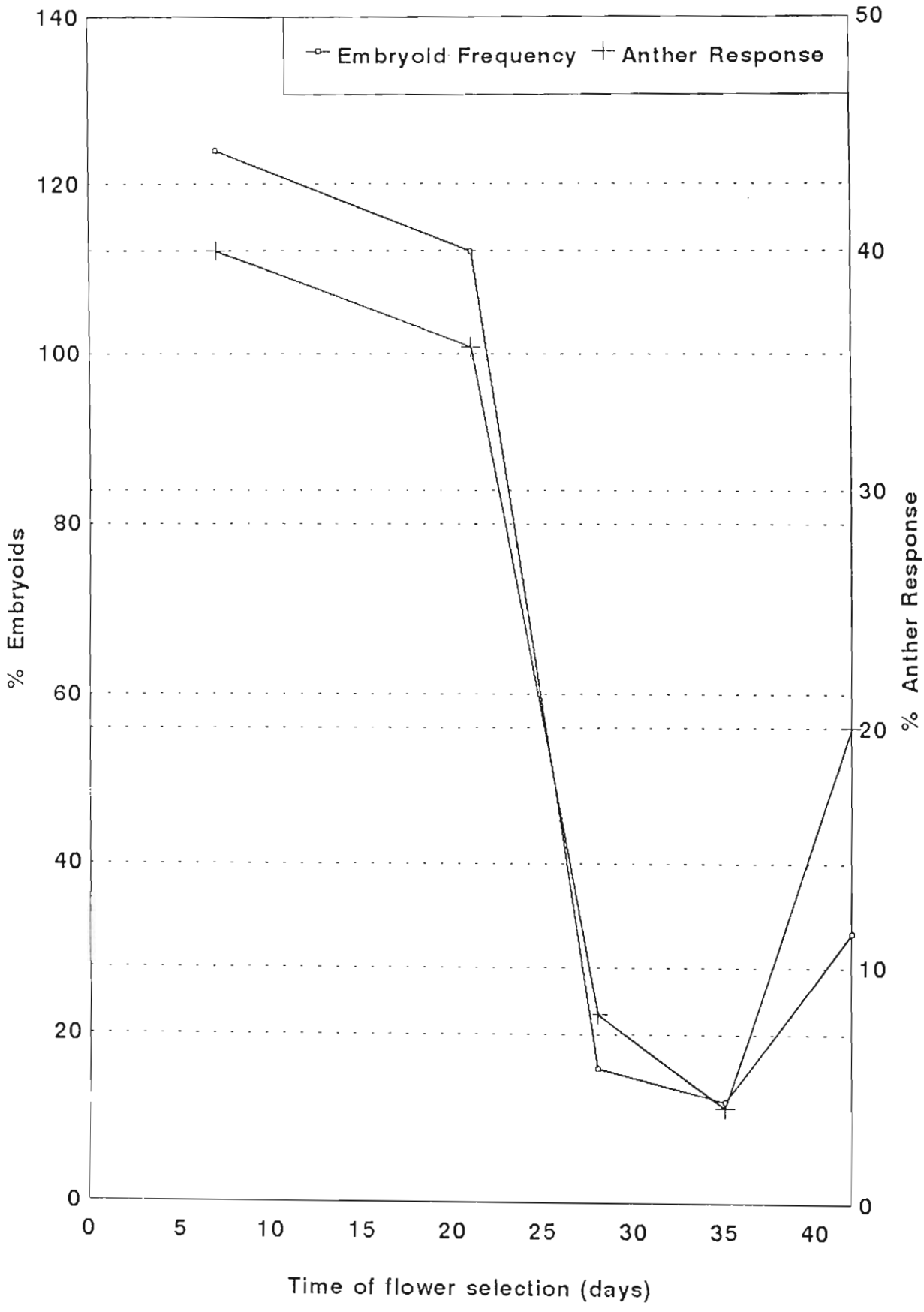


Figure 7 Androgenetic response of anthers of breeding line 87.2002/3 with plant age

Table 6a **Summary of analysis from regression analysis for variable embryoid frequency**

Source	DF	Sum of squares	Mean square	F value
Regression	4	1033	258.29	3.47
Residual	20	1491	74.53	
Corrected Total	24	2524	105.16	

Table 6b **Predictions from regression model between different flowering times for variable embryoid frequency**

Treatment	Embryoid frequency LSMEAN	Standard error LSMEAN	I/J	t-Value 1	2	3	4	5
Time 1	124	43.0	1					
Time 2	112	40.9	2	0.202208				
Time 3	16	15.4	3	2.364558	2.196635			
Time 4	12	13.3	4	2.488342	2.325141	0.196577		
Time 5	32	21.8	5	1.908303	1.726107	0.599457	0.783182	

P = 0.01 t = 2.7874; P = 0.05 t = 2.0595

Time 1 = anthers cultured from flowers harvested 7 days after appearance of first flowers and a 2 day cold pre-treatment;

Time 2 = anthers cultured from flowers harvested 21 days after first flowers and a 2 day cold pre-treatment;

Time 3 = anthers cultured from flowers harvested 28 days after first flowers and a 2 day cold pre-treatment;

Time 4 = anthers cultured from flowers harvested 35 days after first flowers and a 2 day cold pre-treatment;

Time 5 = anthers cultured from flowers harvested 42 days after first flowers and a 2 day cold pre-treatment;

Table 7a **Summary of analysis from regression analysis for variable anther response**

Source	DF	Sum of squares	Mean square	F value
Regression	4	268.1	67.02	2.62
Residual	20	511.6	25.58	
Corrected Total	24	779.7	32.49	

Table 7b **Predictions from regression model between different flowering times for variable anther response**

Treatment	Anther Response LSMEAN	Standard error LSMEAN	i/j	t-Value 1	2	3	4	5
Time 1	40	14.31	1					
Time 2	36	13.57	2	0.202829				
Time 3	8	6.40	3	2.041341	1.866232			
Time 4	4	4.51	4	2.399381	2.237790	0.510892		
Time 5	20	10.12	5	1.141106	0.945176	1.002179	1.444113	

P=0.01 t=2.7874; P=0.05 t=2.0595

Time 1 = anthers cultured from flowers harvested 7 days after appearance of first flowers and a 2 day cold pre-treatment;

Time 2 = anthers cultured from flowers harvested 21 days after first flowers and a 2 day cold pre-treatment;

Time 3 = anthers cultured from flowers harvested 28 days after first flowers and a 2 day cold pre-treatment;

Time 4 = anthers cultured from flowers harvested 35 days after first flowers and a 2 day cold pre-treatment;

Time 5 = anthers cultured from flowers harvested 42 days after first flowers and a 2 day cold pre-treatment;

Table 8a Summary of analysis from regression analysis for variable anther productivity

Source	DF	Sum of squares	Mean square	F value
Regression	4	1058	264.6	1.41
Residual	20	3742	187.1	
Corrected Total	24	4800	200.0	

Table 8b Predictions from regression model between different flowering times for variable anther productivity

Treatment	Anther Productivity LSMEAN	Standard error LSMEAN	i/j	t-Value 1	2	3	4	5
Time 1	240.0	94.8	1					
Time 2	233.3	93.4	2	0.050345				
Time 3	80.0	54.7	3	1.461865	1.416312			
Time 4	60.0	46.8	4	1.702568	1.658863	0.277823		
Time 5	100.0	61.2	5	1.240714	1.193753	0.243657	0.519188	

P=0.01 t=2.7874; P=0.05 t=2.0595

Time = anthers cultured from flowers harvested 7 days after appearance of first flowers and a 2 day cold pre-treatment;
Time 2 = anthers cultured from flowers harvested 21 days after first flowers and a 2 day cold pre-treatment;
Time 3 = anthers cultured from flowers harvested 28 days after first flowers and a 2 day cold pre-treatment;
Time 4 = anthers cultured from flowers harvested 35 days after first flowers and a 2 day cold pre-treatment;
Time 5 = anthers cultured from flowers harvested 42 days after first flowers and a 2 day cold pre-treatment;

Experiment 2: Rapid assessment of microspore developmental stages in potato using DAPI

Although flower bud colour did not differ much between the different flower developmental stages, a definite colour distinction was observed for anther of different anther length. The latter is, however, not a reliable parameter, as it is mere shades of green and/or yellow which are involved and interpretations can consequently greatly vary. Atzimba anthers were, in general, larger than those of 87.2002/3 and *S. canasense* although the anthers in the corresponding groups were in approximately the same microspore developmental stages. This is not unexpected as the flowers from a tetraploid plant are usually bigger than those from a diploid or dihaploid plant.

Non-linear regression was applied to the data (Tables 9a - 11f). First it had to be determined which formulae could best be applied to the results (which formulae fitted the data the best). The different exponential formulae fitted to the data were:

(i) $y = a + b*r^x$ (ordinary exponential formulae);

(ii) $y = a + b*r* + c*s*$ (double exponential);

(iii) $y = a + (b + c*x)*r^x$ (critical exponential);

(iv) $y = a + b*r^x + c*x$ (line plus exponential);

(* = multiplication)

Difficulty was experienced visualize the various microspore developmental stages in the diploid wild species *Solanum canasense* as most microspores were sterile. This also made data analysis difficult. Besides the exponential formulae, described above, rational functions were also fitted to this data. The latter, specifically the quad divided by quad function (QDQ), fitted the data the best. The different rational functions fitted to the data were:

(i) $y = a + b/(1 + d*x)$ (linear divided by linear)

(ii) $y = a + b/(1 + d*x) + c*x$ (quad divided by linear)

(iii) $y = a + (b + c*x)/(1 + d*x + e*x^2)$ (quad divided by quad)

(* = multiplication)

The fitted curve formulae indicated beneath each of Tables 9a - 11f, are the specific ones used. From the data plotted in Figures 8 A-C, it can be concluded that the anthers of smaller flowers contained higher numbers of microspore mother cells, dyads and tetrads which decreased as flower bud length increased. While the opposite proved true for uninucleate and binucleate cells. For breeding line 87.2002/3 (Figure 8A), flower bud lengths of 4.8-6.5 mm contained anthers with the highest number of uninucleate cells. The latter agrees with the results of WENZEL and FOROUGHI-WEHR (1984), who reported an optimal flower bud length of 4-6 mm. The flowers in the mentioned length ranges contained more than 50% microspores in the uninucleate stage of development.

The optimal flower bud length for Atzimba was between 5.15 mm and 7.6 mm, with the highest number of uninucleate microspores contained in anthers of approximately 6.5 mm (Figure 8B). According to the results, a higher concentration of uninucleate microspores occurred at optimum conditions for Atzimba (+80%) compared to that of line 87.2002/3 (+65%). The poor androgenetic response of Atzimba could either be due to the selection of too small flowers or the probability that a later, more advanced developmental stage was required (for instance a late uninucleate or even early to mid-binucleate stage) or that other environmental- and culture conditions are required by this cultivar,

Difficulty was experienced with the determination of the stage of microspore development for the diploid wild species *Solanum canasense* (Figure 8C), as flowers tended to ablactate early in the flowering period of the donor plant and most microspores were sterile. The latter could explain why this genotype showed no amenibilty to any anther culture

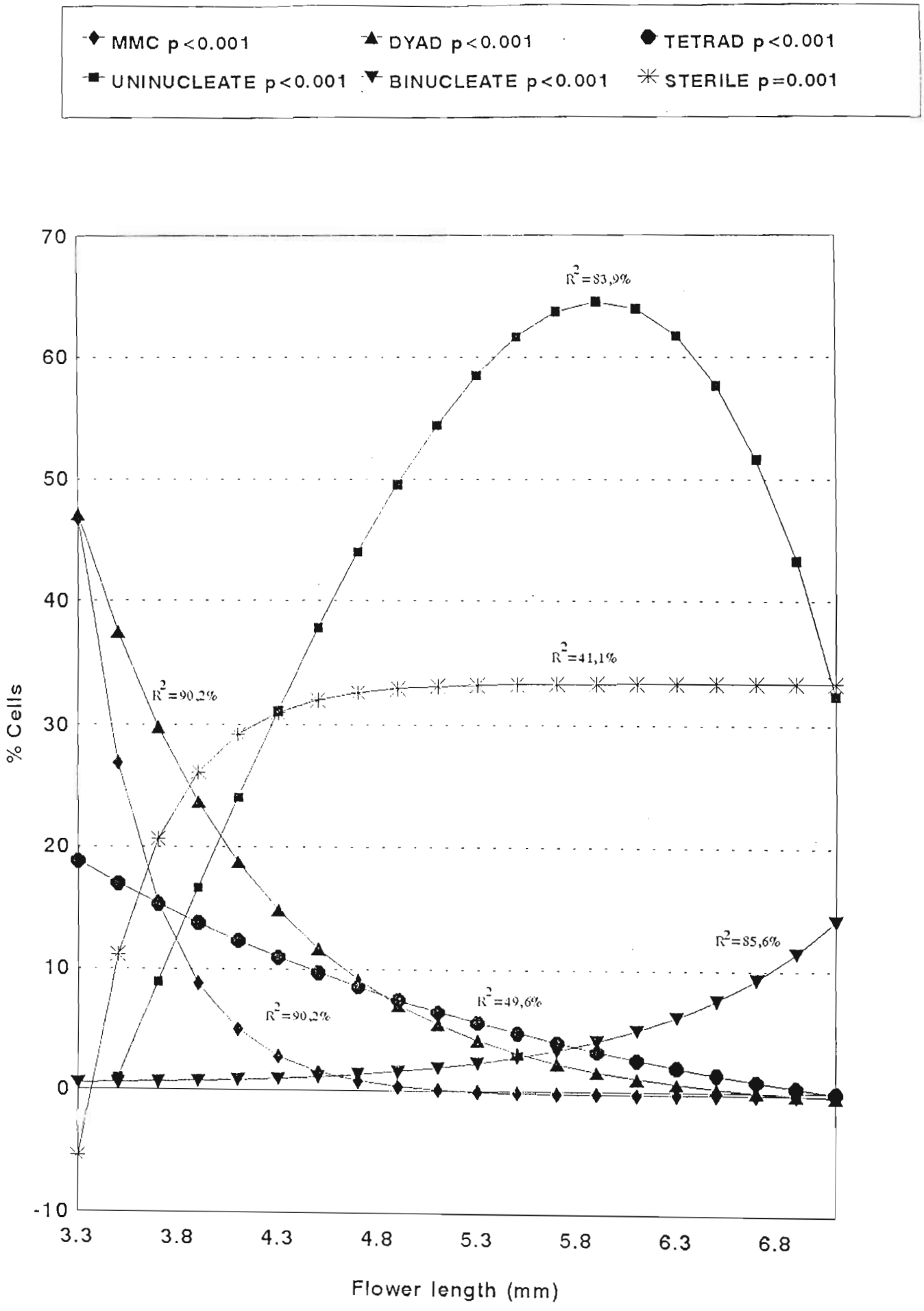


Figure 8A Microspore developmental stages in anthers of dihaploid breeding line 87.2002/3

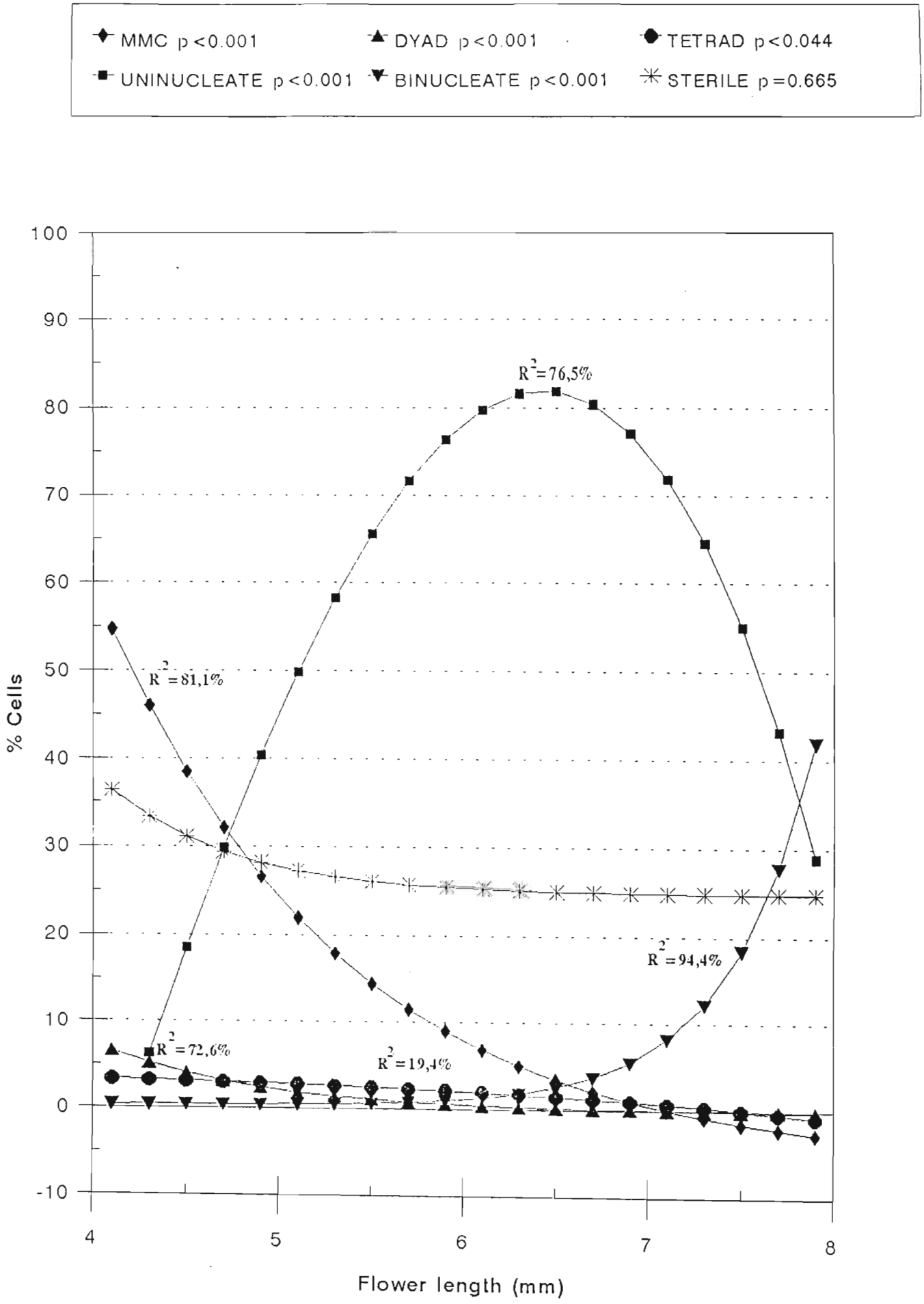


Figure 8B Microspore developmental stages in anthers of tetraploid cultivar Atzimba

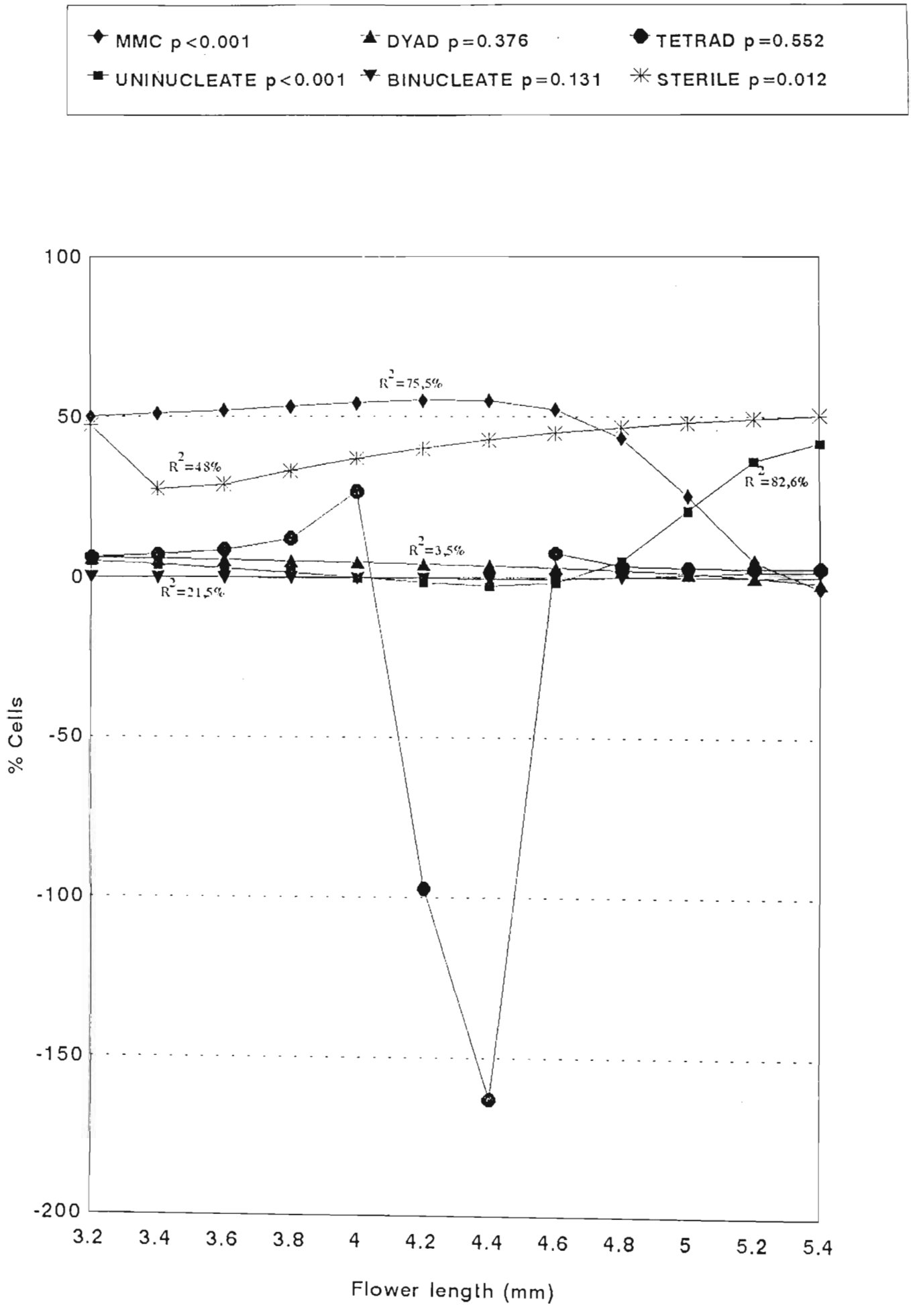


Figure 8C Microspore developmental stages in anthers of diploid wild species Solanum canasense

experiments performed as well as the sharp decrease observed in the number of tetrad cells (Figure 8C). Similar to the results obtained for 87.2002/3 and Atzimba, the smallest flowers of this genotype contained the highest number of microspore mother cells (more than 50% in flowers smaller than 4.4 mm). Contrary to the results obtained for breeding line 87.2002/3, where flowers 3.3 - 4.4 mm in length resulted a sharp decrease in the number of microspore mother cells, the number of these cells still increased in *S. canasense* flowers ranging between 3.2 - 4.4 mm in length. However, a decrease in the number of microspore mother cells and subsequent increase in the number of uninucleate cells were detected in flowers larger than 4.4 mm. The optimal flower bud length for this diploid species correlated more or less with that of breeding line 87.2002/3, namely between 5.0 - 5.4 mm and larger. For this study, however, flowers larger than 5.4 mm could not be selected, due to the fact phenomenon that most flowers tend to ablactate.

From the results it can be seen that the optimal flower bud length required for anthers to be in the uninucleate stage of microspore development differed, especially between the tetraploid genotype compared to the diploid and dihaploid genotypes. This agrees with results from KASPERBAUER and COLLINS (1974) and PIERIK (1993) who reported that the correlation between flower bud size and the stage of microspore development can differ between cultivars, due to genetic differences. To assure maximum response in anther cultures of responsive lines, it is recommended to first determine the optimal flower bud length as well as pollen fertility before commencing with any *in vitro* experiments. Furthermore, cells/microspores at different developmental stages (eg. micrspore mother cells, diades and even tetrads) were present within the same anther. This supports the results of PIERIK (1993), namely that microspore development is not synchronized within an anther. It also indicates that the microspores are viable and in the process of active mitosis.

Table 9a **Summary of non-linear regression analysis for variable microspore mother cells for line 87.2002/3**

Source	DF	Sum of squares	Mean square	Pr > F
Regression	2	5287.6	2643.80	0.001
Residual	21	522.2	24.86	
Corrected Total	23	5809.7	252.60	

Fitted curve formulae: $Y = A + B*R^x$; $R^2=90.2$

Estimates of parameters	Estimate	s.e.
R	0.0643	0.0346
B	402496.	725908
A	-0.21	1.32

Table 9b **Summary of analysis from non-linear regression analysis for variable dyad cells for line 87.2002/3**

Source	DF	Sum of squares	Mean square	Pr > F
Regression	2	5980.0	2989.99	0.001
Residual	21	590.0	28.14	
Corrected Total	23	6570.9	285.69	

Fitted curve formulae: $Y = A + B*R^x$; $R^2=90.2$

Estimates of parameters	Estimate	s.e.
R	0.3305	0.073
B	1858.0	1341.
A	-1.06	2.59

Table 9c **Summary of analysis from non-linear regression analysis for variable tetrad cells for line 87.2002/3**

Source	DF	Sum of squares	Mean square	Pr > F
Regression	2	838.9	419.46	0.001
Residual	19	703.5	37.03	
Corrected Total	21	1542.4	73.45	

Fitted curve formulae: $Y = A + B*R^x$; $R^2 = 49.6$

Estimates of parameters	Estimate	s.e.
R	0.680	0.27
B	87.6	77.4
A	-5.7	14.0

Table 9d **Summary of analysis from non-linear regression analysis for variable uninucleate cells for line 87.2002/3**

Source	DF	Sum of squares	Mean square	Pr > F
Regression	2	12469.0	6234.3	0.001
Residual	21	3682.0	175.4	
Corrected Total	23	16151.0	702.2	

Fitted curve formulae: $Y = A + (B + C*X)*R^x$; $R^2 = 83.9$

Estimates of parameters	Estimate	s.e.
R	0.341	0.128
B	-2420.0	2936.
A	61.95	6.65

Table 9e **Summary of analysis from non-linear regression analysis for variable binucleate cells for line 87.2002/3**

Source	DF	Sum of squares	Mean square	Pr > F
Regression	2	366.17	183.09	0.001
Residual	21	55.43	2.64	
Corrected Total	23	421.60	18.33	

Fitted curve formulae: $Y = A + B*R^x$; $R^2=85.6$

Estimates of parameters	Estimate	s.e.
R	2.917	0.829
B	0.0069	0.013
A	-0.301	0.776

Table 9f **Summary of analysis from non-linear regression analysis for variable sterile cells for line 87.2002/3**

Source	DF	Sum of squares	Mean square	Pr > F
Regression	2	3518.0	1759.1	0.001
Residual	21	4098.0	195.2	
Corrected Total	23	7617.0	331.2	

Fitted curve formulae: $Y = A + B*R^x$; $R^2=41.1$

Estimates of parameters	Estimate	s.e.
R	0.062	0.116
B	-375227	2350148
A	33.42	3.69

Table 10a **Summary of analysis from non-linear regression analysis for variable microspore mother cells for cultivar Atzimba**

Source	DF	Sum of squares	Mean square	Pr > F
Regression	2	8647.0	4323.27	0.001
Residual	21	1725.0	82.15	
Corrected Total	23	10372.0	450.94	

Fitted curve formulae: $Y = A + B*R^x$; $R^2=81.8$

Estimates of parameters	Estimate	s.e.
R	0.458	0.110
B	1487.0	1407
A	-5.78	5.98

Table 10b **Summary of analysis from non-linear regression analysis for variable dyad cells for cultivar Atzimba**

Source	DF	Sum of squares	Mean square	Pr > F
Regression	2	86.64	43.322	0.001
Residual	20	28.76	1.438	
Corrected Total	22	115.40	5.245	

Fitted curve formulae: $Y = A + B*R^x$; $R^2=72.6$

Estimates of parameters	Estimate	s.e.
R	0.303	0.117
B	898.0	1452
A	-0.134	0.490

Table 10c **Summary of analysis from non-linear regression analysis for variable tetrad cells for cultivar Atzimba**

Source	DF	Sum of squares	Mean square	Pr > F
Regression	2	41.4	20.701	0.044
Residual	20	113.3	5.664	
Corrected Total	22	154.7	7.031	

Fitted curve formulae: $Y = A + B*R^x$; $R^2 = 19.4$

Estimates of parameters	Estimate	s.e.
R	1.55	1.23
B	-0.16	1.10
A	4.31	**4.47**

Table 10d **Summary of analysis from non-linear regression analysis for variable uninucleate cells for cultivar Atzimba**

Source	DF	Sum of squares	Mean square	Pr > F
Regression	2	10740.0	5369.9	0.001
Residual	21	12367.0	588.9	
Corrected Total	23	23107.0	1004.7	

Fitted curve formulae: $Y = A + (B + C*X)*R^x$; $R^2 = 76.5$

Estimates of parameters	Estimate	s.e.
R	0.188	0.192
B	-63518.0	269169
A	58.26	7.69

Table 10e **Summary of analysis from non-linear regression analysis for variable binucleate cells for cultivar Atzimba**

Source	DF	Sum of squares	Mean square	Pr > F
Regression	2	4665.1	2332.54	0.001
Residual	21	251.6	11.98	
Corrected Total	23	4916.7	213.77	

Fitted curve formulae: $Y = A + B*R^x$; $R^2 = 94.4$

Estimates of parameters	Estimate	s.e.
R	8.05	2.07
B	0.000002	0.00000
A	0.367	0.972

Table 10f **Summary of analysis from non-linear regression analysis for variable sterile cells for cultivar Atzimba**

Source	DF	Sum of squares	Mean square	Pr > F
Regression	2	323.0	161.5	0.665
Residual	21	8155.0	388.3	
Corrected Total	23	8478.0	368.6	

Fitted curve formulae: $Y = A + B*R^x$;
Residual variance exceeds variance of Y variate

Estimates of parameters	Estimate	s.e.
R	0.217	0.948
B	6098.0	110519
A	24.77	6.58

Table 11a **Summary of non-linear regression analysis for variable microspore mother cells for *S. canasense***

Source	DF	Sum of squares	Mean square	Pr > F
Regression	4	7944	1986.1	0.001
Residual	13	18352	141.2	
Corrected Total	17	9780	575.3	

Fitted curve formulae: $Y = A + (B + C*X)/(1 + D*X + E*X^2)$;
$R^2 = 75.5$

Estimates of parameters	Estimate	s.e.
D	-0.3875	0.0162
E	0.03793	0.0032
B	5.17	2.55
C	-1.062	0.508
A	38.17	9.68

Table 11b **Summary of analysis from non-linear regression analysis for variable dyad cells for *S. canasense***

Source	DF	Sum of squares	Mean square	Pr > F
Regression	4	87.9	21.98	0.376
Residual	13	247.8	19.06	
Corrected Total	17	335.7	19.75	

Fitted curve formulae: $Y = A + (B + C*X)/(1 + D*X + E*X^2)$;
$R^2 = 3.5$

Estimates of parameters	Estimate	s.e.
D	-0.293	0.931
E	0.021	0.164
B	-3.1	71.4
C	0.5	12.3
A	11.3	93.5

Table 11c **Summary of analysis from non-linear regression analysis for variable tetrad cells for *S. canasense***

Source	DF	Sum of squares	Mean square	Pr > F
Regression	4	91.4	22.86	0.552
Residual	13	375.8	28.91	
Corrected Total	17	467.3	27.49	

Fitted curve formulae: $Y = A + (B + C*X)/(1 + D*X + E*X^2)$;
Residual variance exceeds variance of Y variate

Estimates of parameters	Estimate
D	-0.4673
E	0.05454
B	0.4829
C	-0.1012
A	3.745

Table 11d **Summary of analysis from non-linear regression analysis for variable uninucleate cells for *S. canasense***

Source	DF	Sum of squares	Mean square	Pr > F
Regression	4	4333.4	1083.35	< 0.001
Residual	13	664	51.08	
Corrected Total	17	4997.4	293.97	

Fitted curve formulae: $Y = A + (B + C*X)/(1 + D*X + E*X^2)$;
$R^2 = 82.6$

Estimates of parameters	Estimate
D	-0.3935
E	0.03909
B	-4.231
C	0.8559
A	15.6

Table 11e **Summary of analysis from non-linear regression analysis for variable binucleate cells for *S. canasense***

Source	DF	Sum of squares	Mean square	Pr > F
Regression	4	8.67	2.166	0.131
Residual	13	13.03	1.003	
Corrected Total	17	21.7	1.277	

Fitted curve formulae: $Y = A + (B + C*X)/(1 + D*X + E*X^2)$; $R^2 = 21.5$

Estimates of parameters	Estimate
D	-0.4
E	0.04021
B	-0.1407
C	0.02881
A	0.3324

Table 11f **Summary of analysis from non-linear regression analysis for variable sterile cells for *S. canasense***

Source	DF	Sum of squares	Mean square	Pr > F
Regression	4	1571	392.78	0.012
Residual	13	1038	79.88	
Corrected Total	17	2610	153.5	

Fitted curve formulae: $Y = A + (B + C*X)/(1 + D*X + E*X^2)$; $R^2 = 48.0$

Estimates of parameters	Estimate	s.e.
D	-0.67	0.454
E	0.113	0.138
B	12.9	62.9
C	-4.1	19.6
A	64.1	33.1

Experiment 3: Assessment of the mode of microspore development and embryoid formation in cultured potato anthers by means of microtome sections.

3.1 The anatomy of potato anthers

Each anther contains four pollen sacs (ps) which are paired in two anther lobes. The two lobes are connected by sterile connective tissue (ct) through which a vascular bundle (VB) passes. With the degeneration of the connective tissue, the two pollen sacs present in each lobe fuse. The anther wall consists of the following layers (from the most outer layer inwards): the cuticle, epidermis (E), endothecium (En), middle layer (ml) and tapetum (Tp). Based on the behaviour of secondary parietal layers, DAVIS (1966) (as quoted from JOHRI, 1984) recognized four types of anther wall, namely the basic type, dicotyledonous, monocotyledonous and reduced types. Potato anthers have a basic type of anther wall. Here the outer and inner secondary parietal layers divide periclinally and form the endothecium, two middle layers and the tapetum.

The epidermis consists of a single cell layer. In the region of dehiscence, the epidermal cells are quite small and they easily rupture when the anther ripens. Two to four layers of endothecium cells can occur. The middle layer initially consists of a single cell layer, which divides both periclinally and anticlinally to later consist of two layers. During later developmental stages this middle layer to degenerates.

The formation of the tapetum takes place as a result of gradual differentiation in the anther (FAHN, 1974). The tapetum has a nutritive role and potato anthers possess a *secretory-type* tapetum. Thus, the tapetal cells remain in their original position where they later disintegrate and their contents are absorbed by the microspore mother cells and developing microspores (FAHN, 1974). The anther wall bursts open for

microspore dehiscence by means of a longitudinal slit, namely the stomium (St). This opening mechanism was described by FAHN (1974) as follows: The endothecium is situated immediately below the epidermis. U-shaped thickenings, with their gaps directly towards the epidermis, develop in the endothecium shortly before the liberation of the developed microspores. During dehydration of the anther the endothecium loses water, with the result that the cell walls of these endothecium cells are drawn towards its centre. The latter is due to the cohesion forces between water molecules and the adhesion forces between the water and the cell walls (FAHN, 1974). As the endothecium thickenings (or endothecium bands, EB) only form in the inner periclinal and anticlinal walls and not in the outer periclinal walls, the outer walls becomes more folded, appearing trapezium-shaped in cross-section. Following water loss, all the external walls fold and wrinkle and the endothecium shrinks in such a manner that it results in the opening of the anther wall. The stomium cells in the region of dehiscence become elongated and thin-walled. Prior to dehiscence, a part of the partition between the two pollen sacs of a lobe usually disintegrates and this region is then covered only by the epidermis (FAHN, 1974).

Due to the poor response of *S. canasense* and Atzimba, it was decided to study only line 87.2002/3 completely; that is sections from the 10 different treatments described in the experimental outline. Sections of the first, second, seventh and tenth treatments were studied of Atzimba and *S. canasense* material. Flowers of varying lengths, ranging between 3 - 6 mm, were selected at random.

Microspores are unicellular cells. As soon as cell- or nuclear division occurred, they are no longer referred to as microspores but young, developing pollen (or microgametophytes). In the following discussion the term "microspore(s)" is used, however, to refer to both the unicellular and multicellular phase - as it is shorter and easier to follow.

3.2 Interpretation of microtome sections of the anthers of breeding line 87.2002/3

3.2.1 Treatment 1:

Material for this treatment were fixed immediately after harvest, without a cold pre-treatment or a culture period on nutrient medium. Most of the microspores were in the uninucleate stage of development, although both the early and late binucleate stages were also present (Figure 9.1). The late binucleate stage of microspore development was characterized by the presence of prominent nucleoli in some microspores (Figures 9.1 A and B). Due to the occurrence of both the uni- and binucleate developmental stages, it can be concluded that the microspores were in the process of mitosis. The microspores were almost round with a well developed tapetum. Uninucleate connective tissue surrounded the pollen sacs. In all microtome sections studied, the four pollen sacs were not fused. While the stomium could be distinguished, it was still closed.

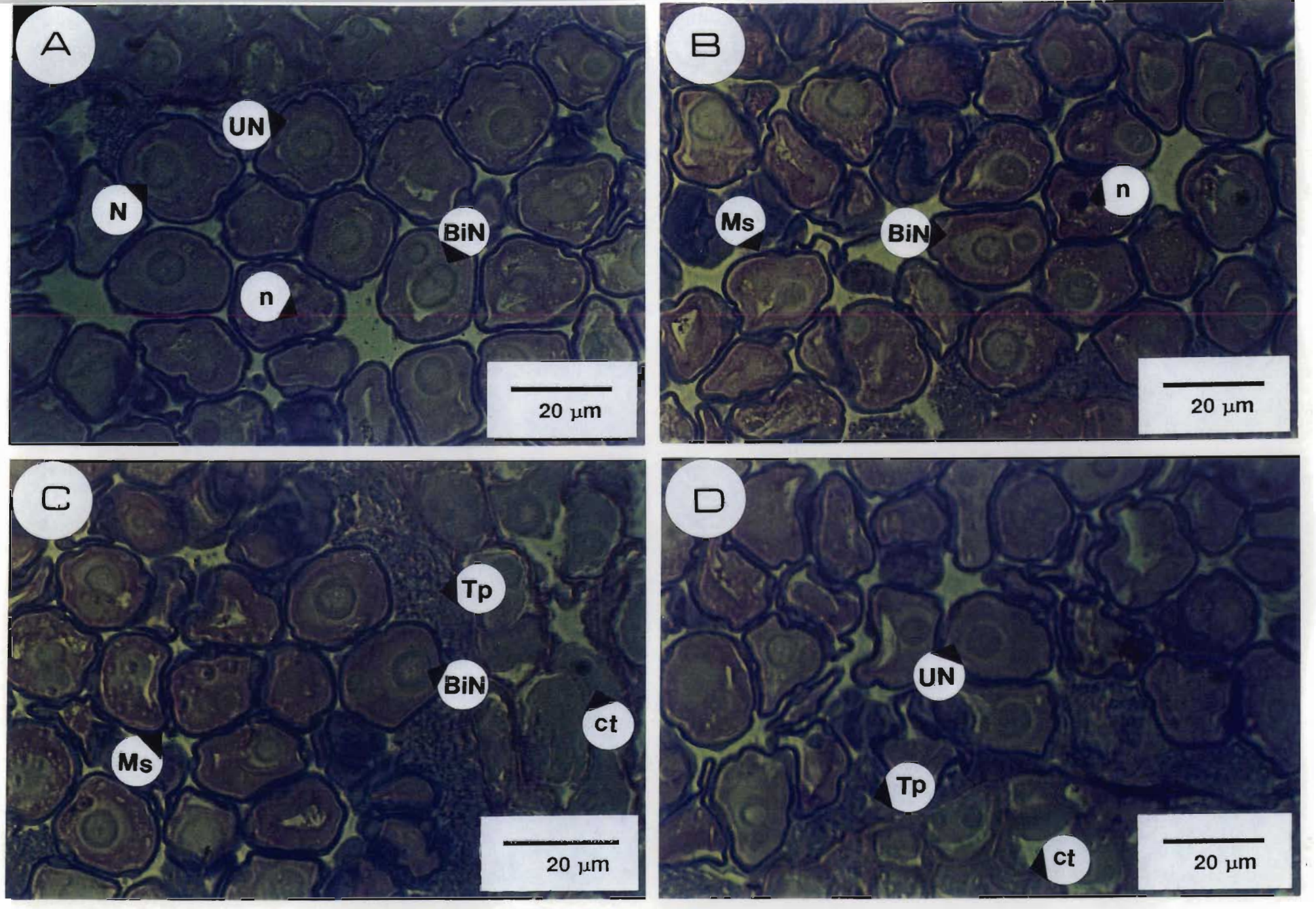
3.2.2 Treatment 2:

In most sections studied the microspores were round and their overall appearance did not differ much from those in Treatment 1, despite the fact that these anthers received a cold pre-treatment of two days before fixation. Uninucleate, binucleate as well as a few sterile microspores were present in these anthers and the tricolpate sculpture of the pollen sporoderm could be distinguished (Figures 9.2 A - D). The occurrence of the different microspore developmental stages again confirmed the process of mitosis (as in Treatment 1). Some of the microspores contained one or more vacuoles (Figures 9.2 C and D). The microspores shown in Figures 9.2 A and B differed both in shape and in the number of sterile microspores compared to that in Figures 9.2 C and D. Thus, the four anthers from the same treatment and selected from the same conditions differed greatly from each other.

Figure 9.1 Cross-section of 87.2002/3 anthers fixed immediately after harvest, without a cold pre-treatment and without culturing on nutrient medium (Treatment 1) to indicate:

A - D. Uninucleate and binucleate microspores. Late binucleate stage also occurred as well developed nucleoli could be distinguished. All microspores were approximately round in shape with a thick, well developed tapetum and uninucleate connective tissue.

BiN, binucleate microspores; **ct**, connective tissue; **Ms**, microspores; **n**, nucleolus; **N**, nucleus; **Tp**, tapetum; **UN**, uninucleate microspores.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BiN
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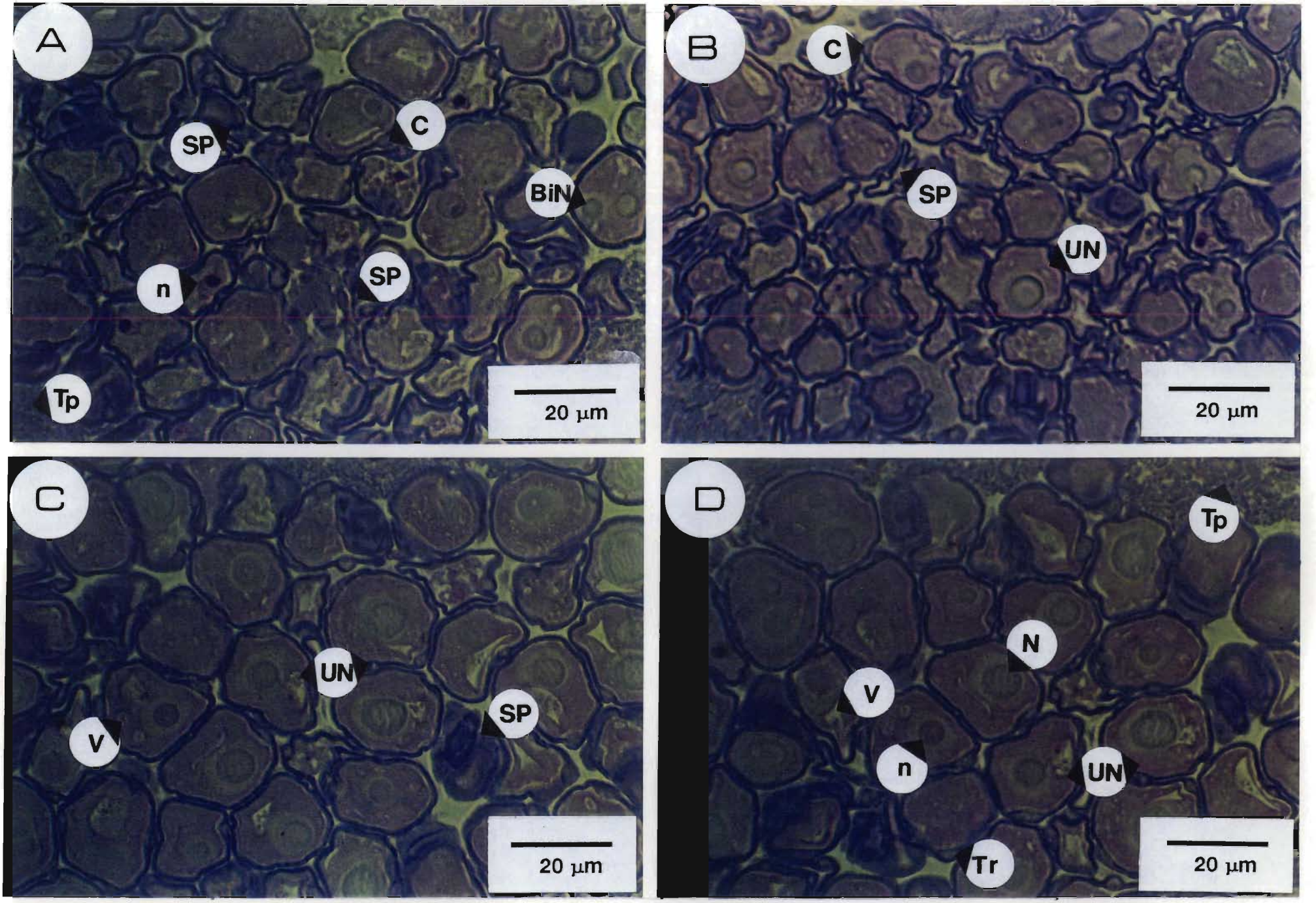
Tp
BiN
ct
Ms
20 µm
D
UN
Tp
ct
20 µm

Figure 9.2 Cross-section of 87.2002/3 anthers fixed after a cold pre-treatment of 2 days but without culturing on nutrient medium (Treatment 2) to indicate:

A - B. Microspores in the uninucleate and binucleate development stage as well as large amounts of sterile pollen. The tapetum was still thick and well developed and a tricolpate microspore (only one colpus distinguishable here) occurred.

C - D. Microspores still had a round shape which did not differ much from those of Treatment 1. Some microspores also contained small vacuoles.

BiN, binucleate microspores; **C**, colpi(us); **n**, nucleolus; **N**, nucleus; **SP**, sterile pollen; **Tp**, tapetum; **Tr**, tricolpate pollen; **UN**, uninucleate microspores; **V**, vacuole.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3.2.3 Treatment 3:

As soon as anthers were exposed to culture medium the microspores lost their round shape as they started to either develop or degenerate (sterile microspores). Both mature uninucleate microspores, surrounded by the *intine* and outer *exine* layers, and sterile microspores were observed (Figure 9.3 i). Tricolpate pollen sporoderms were also visible in many microspores. In some microtome sections (Figures A and B) multicellular microspores were present, indicating the first stages of embryoid differentiation. Therefore, it can be concluded that the earliest embryogenic development occurred after a culture period of seven days. In correlation with this development, the secretory tapetum also started to degenerate (Figures A and C), confirming the tapetum's nutritive role in microspore development. Due to the occurrence of both uninucleate and multicellular microspores within the same anther and even the same pollen sac, it can be concluded that the developmental stages of microspores contained in an anther, is not synchronized. This correlates with the results of PIERIK (1993).

Figure 9.3 (ii) E represents an almost complete cross-section of an anther, indicating the four pollen sacs (ps) (still not fused) the vascular bundle (VB), epidermis (E), connective tissue (ct), middle layer (ml) degenerating tapetum (Tp) and endothecium (En). At high magnification (Figure F) the endothecium bands (EB) situated in the bottom layers of the endothecium can be seen, as well as the stomium (St) and elongated stomium cells in the region where dehiscion should occur (Figure G).

Figure 9.3 (i) Cross-section of 87.2002/3 anthers fixed after a cold pre-treatment of 2 days and a culture period of 7 days on nutrient medium (Treatment 3) to indicate:

A - B. Mature uninucleate microspores enclosed within an inner layer, the intine (pink), and an outer layer, the exine (blue). The tapetum was still thick and well developed. Sterile pollen also occurred. Well developed multicellular microspores (with approximately 4 cells, divided by cell plates, distinghuisable) were also observed at this stage.

C. Mature uninucleate microspores surrounded by both intine and exine. On a large number of microspores the tricolpate structure of the pollen sporoderm was well defined and easily distinguishable. The secretorial tapetum started to degenerate.

D. A large number of mature, uninucleate microspores as well as sterile pollen occurred. Most microspores lost their round shape and either degenerated or developed into mature microspores.

cp, cell plate(s); **C**, colpi(us); **ex**, exine; **in**, intine; **Mu**, multicellular microspores (young, developing embryoids); **N**, nucleus; **SP**, sterile pollen; **Tp**, tapetum; **Tr**, tricolpate microspores; **UN**, uninucleate microspores.

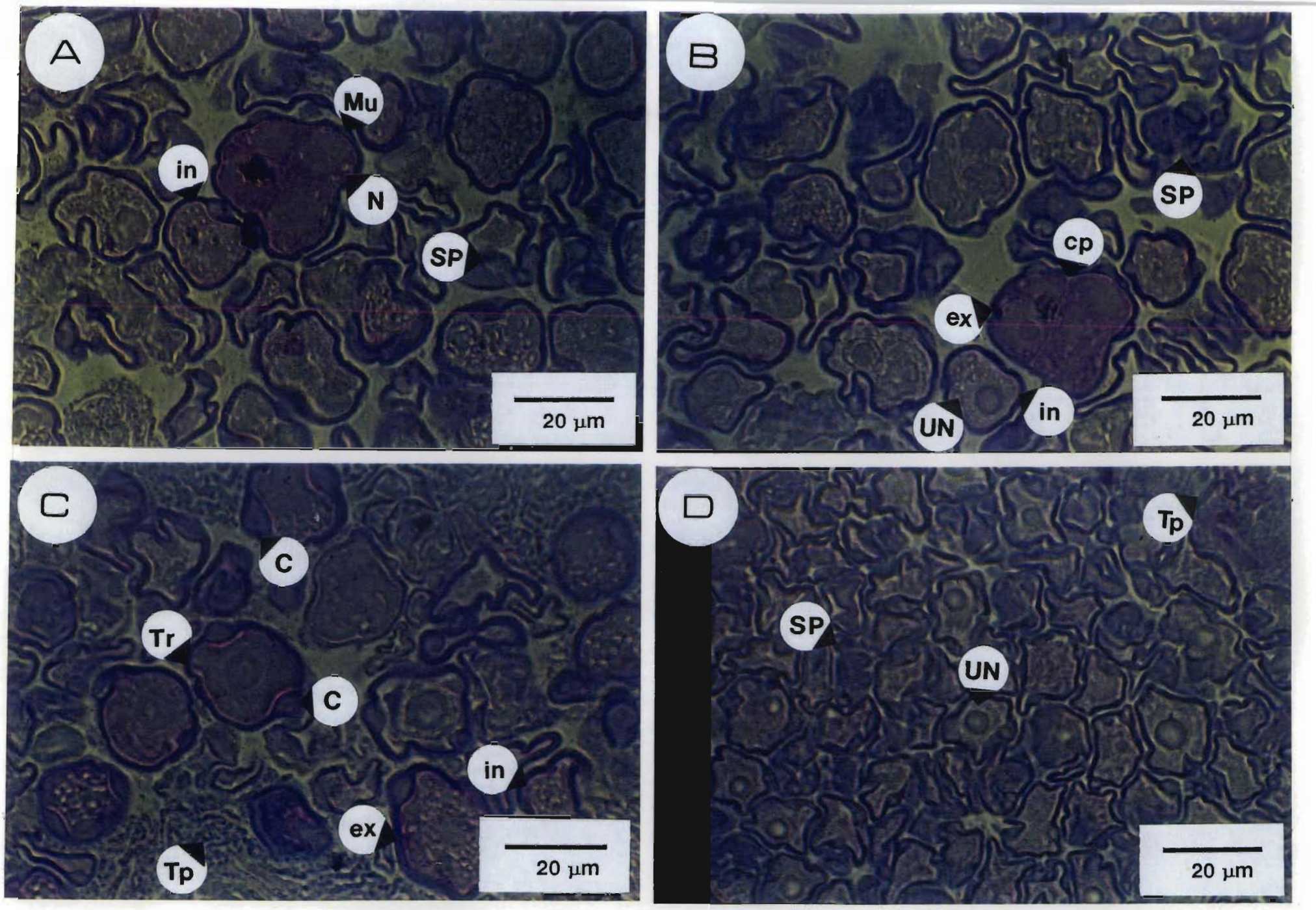
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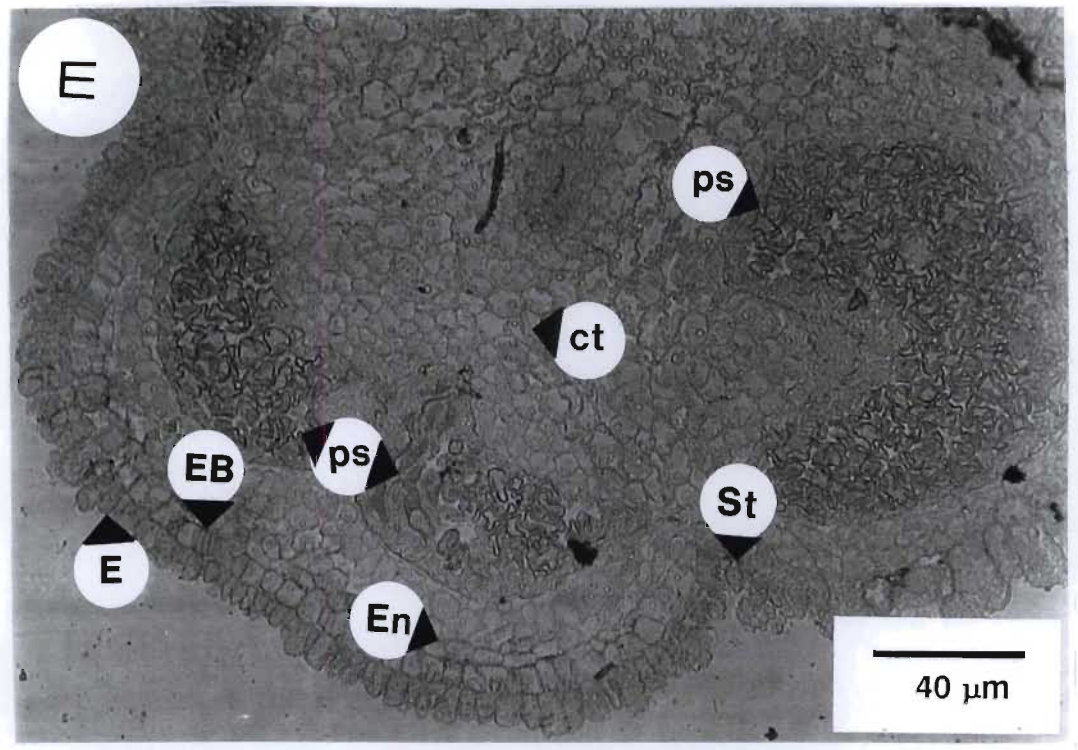
Figure 9.3 (ii) Cross-section of 87.2002/3 anthers fixed after a cold pre-treatment of days and a culture period of 7 days on nutrient medium (Treatment 3) to indicate:

E. Section of anther indicating the endothecium bands situated in the endothecium layers at the bottom part of the anther.
F. Enlargement of Figure E showing the endothecium bands.
G. Elongated, thin-walled cells of the connective tissue in the region where anther will dehisce following opening of the stomium.

ct, connective tissue; **E**, epidermis; **EB**, endothecium bands; **En**, endothecium; **ml**, middle layer; **MS**, microspores; **ps**, pollen sac; **St**, stomium; **Tp**, tapetum.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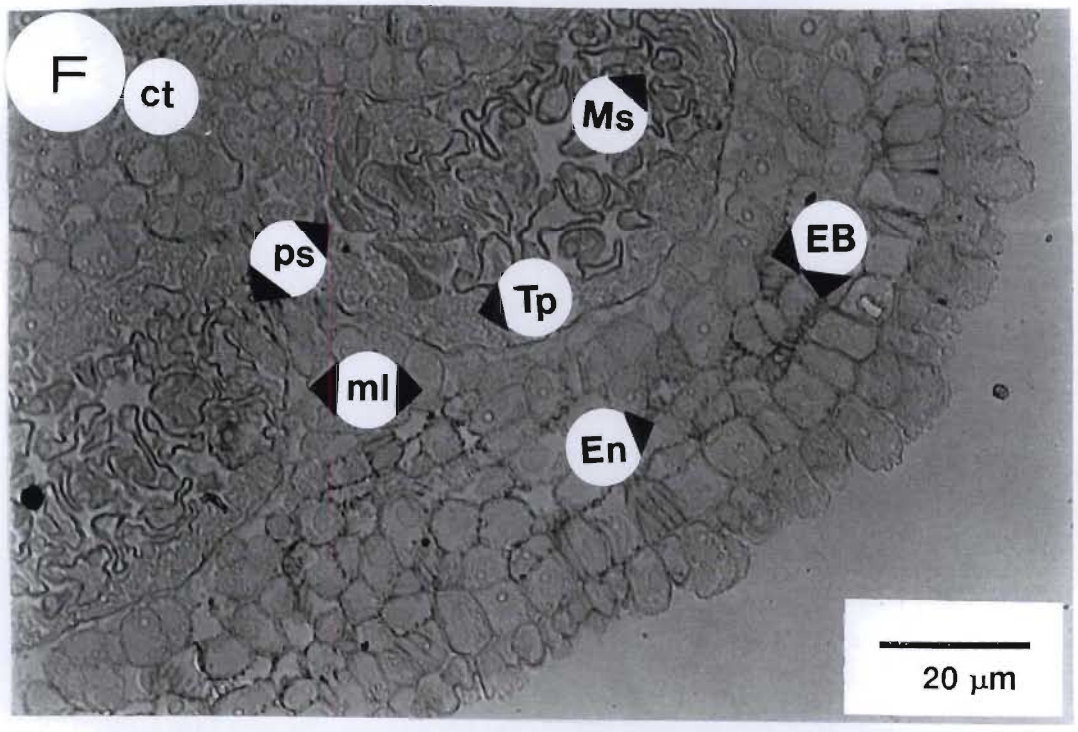
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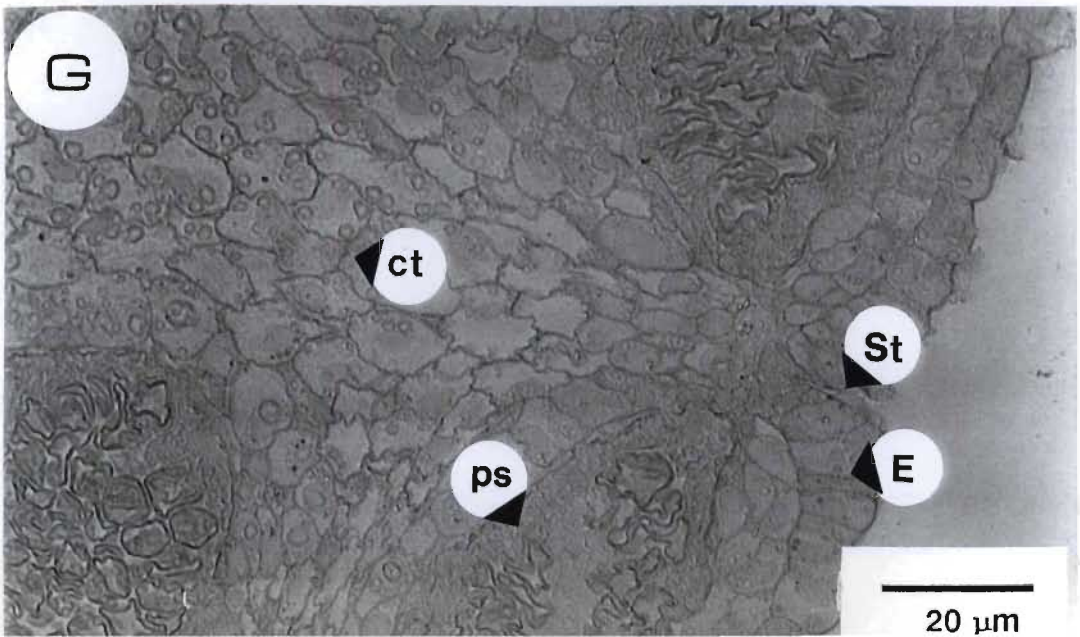
20 µm


G
ct
St
ps
E
20 µm

3.2.4 Treatment 4:

Anthers of this treatment were cultured for 14 days (Figures 9.4 A - K). Although the pollen sacs were not yet fused and the stomia remained intact (as in Treatment 3), **optimal microspore development** of fertile microspores amenable to *in vitro* embryogenesis, occurred at this stage. A large number of multicellular microspores (young, developing embryoids) - still enclosed within the pollen sporoderm - were seen, as were sterile microspores and embryogenic callus. The embryogenic callus appeared as an undifferentiated cell-mass and was already released from the pollen sporoderms at this stage (Figures 9.4 A, B, E and H). However, the majority of developing embryoids were still enclosed within the thick, nourishing walls of the microspores (intine and exine). In a few cases - exine rupture were just started.

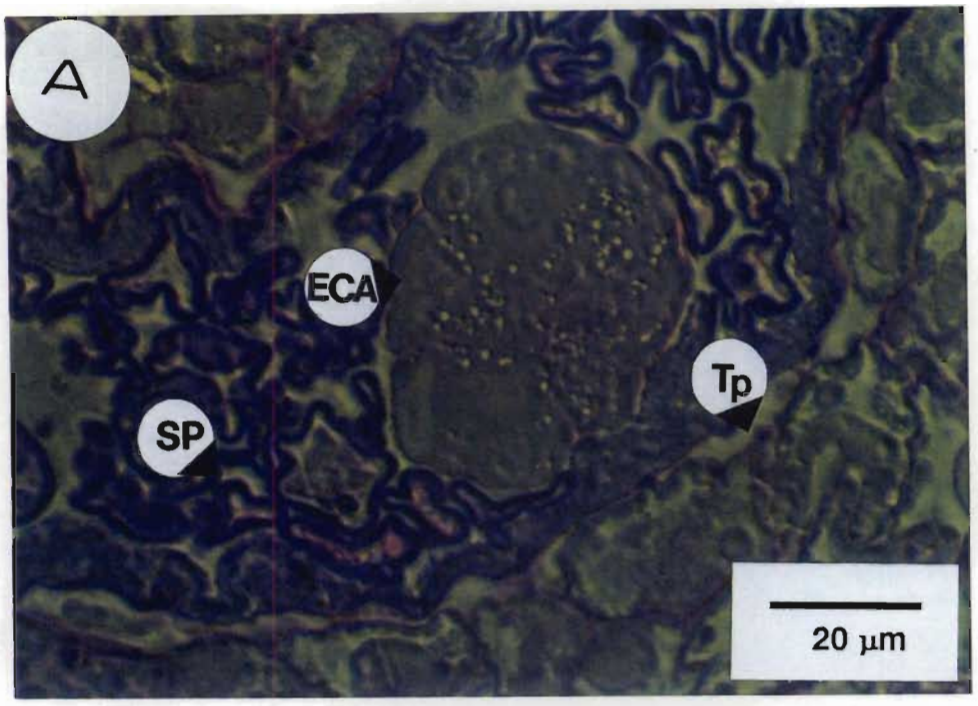
The tapetum was thin and highly degenerated due to its increasing nutritive role. From the latter it can be concluded that the anthers of breeding line 87.2002/3, posessed an efficient nutrient supply- and transfer system, which could be one of the reasons for its high androgenetic response *in vitro*.

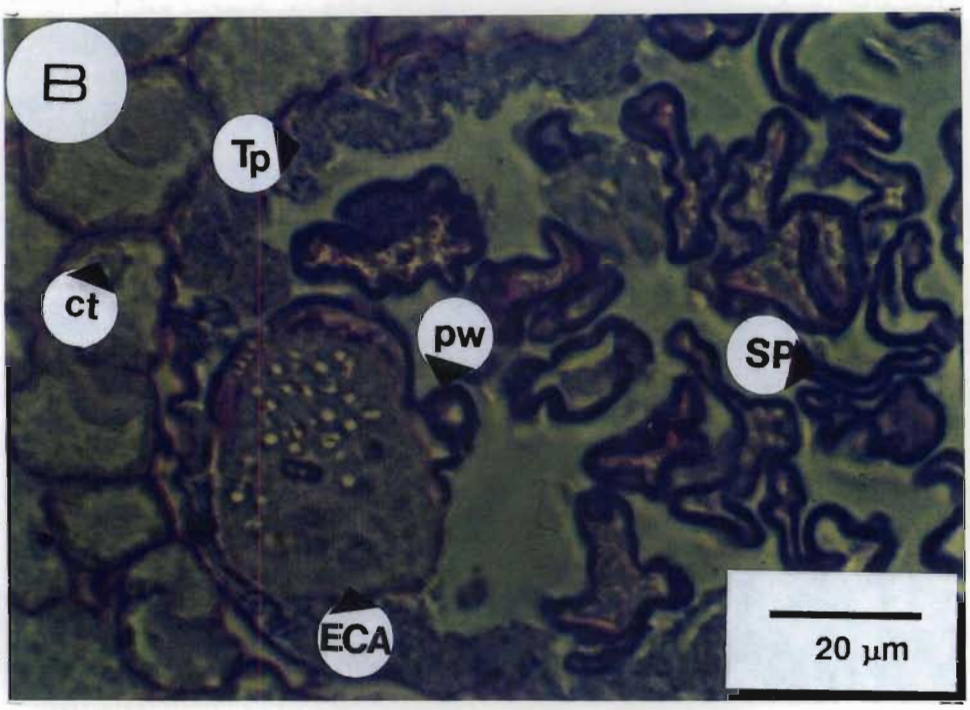
Figure 9.4 (i) Cross-section of 87.2002/3 anthers fixed after a cold pre-treatment of 2 days and a culture period of 14 days on nutrient medium (Treatment 4) to indicate:

A - B. Consecutive sections from the same anther but in different pollen sacs: Thin, degenerating tapetum. Large numbers of sterile pollen. Well developed embryogenic callus, already released from the pollen grain. The remainder of the pollen sporoderm from which the callus have been released, clearly visible.

C. A large number of sterile pollen, uninucleate connective tissue and largely degenerated tapetum. Multicellular microspores (young, developing embryoids) consisting of clearly differentiated cell plates and a single nucleus present in each cell. Photo's C, F and G are microtome sections of the same anther.)

cp, cell plate(s); **ct**, connective tissue; **ECA**, embryogenic callus; **Mu**, multicellular microspores (young, developing embryoids); **N**, nucleus; **pw**, pollen sporoderm; **SP**, sterile pollen; **Tp**, tapetum.


A
ECA
Tp
SP
20 µm


B
Tp
ct
pw
SP
ECA
20 µm

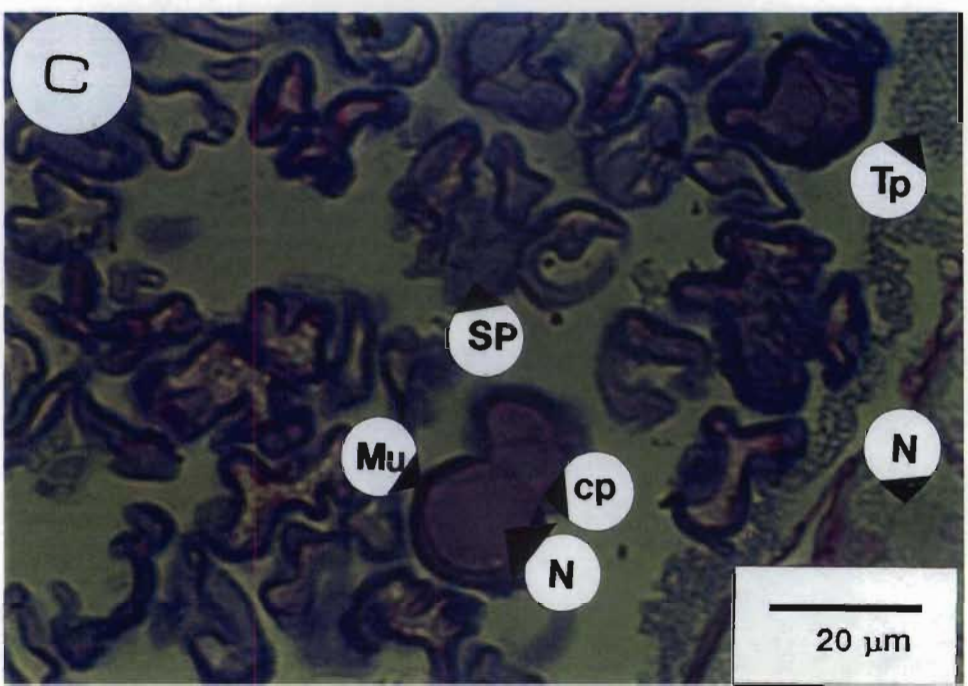
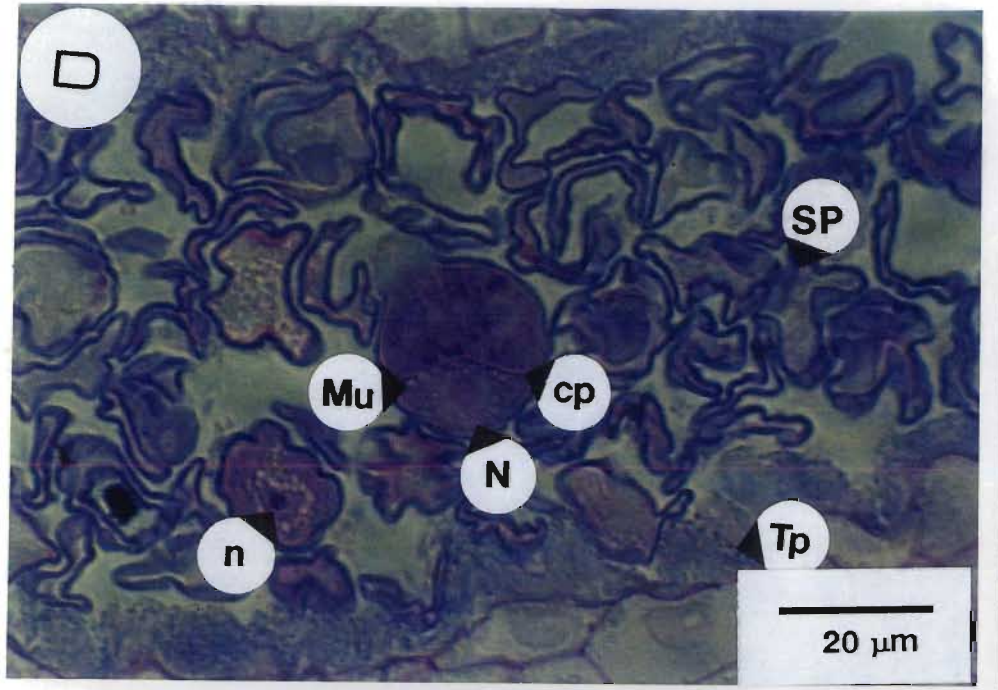
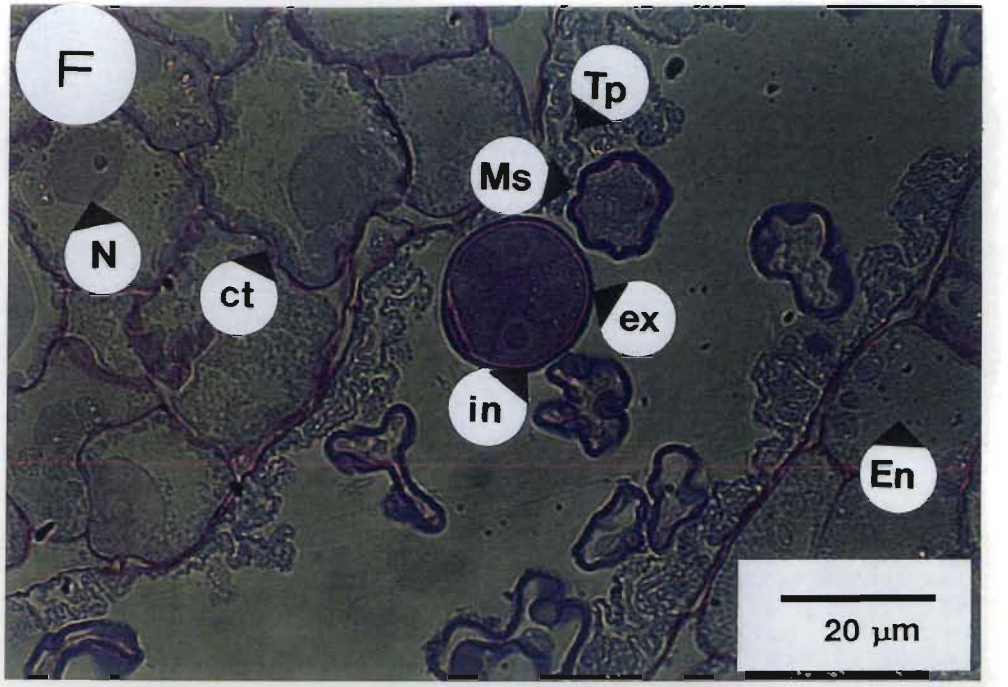

C
Tp
SP
Mu
cp
N
N
20 µm

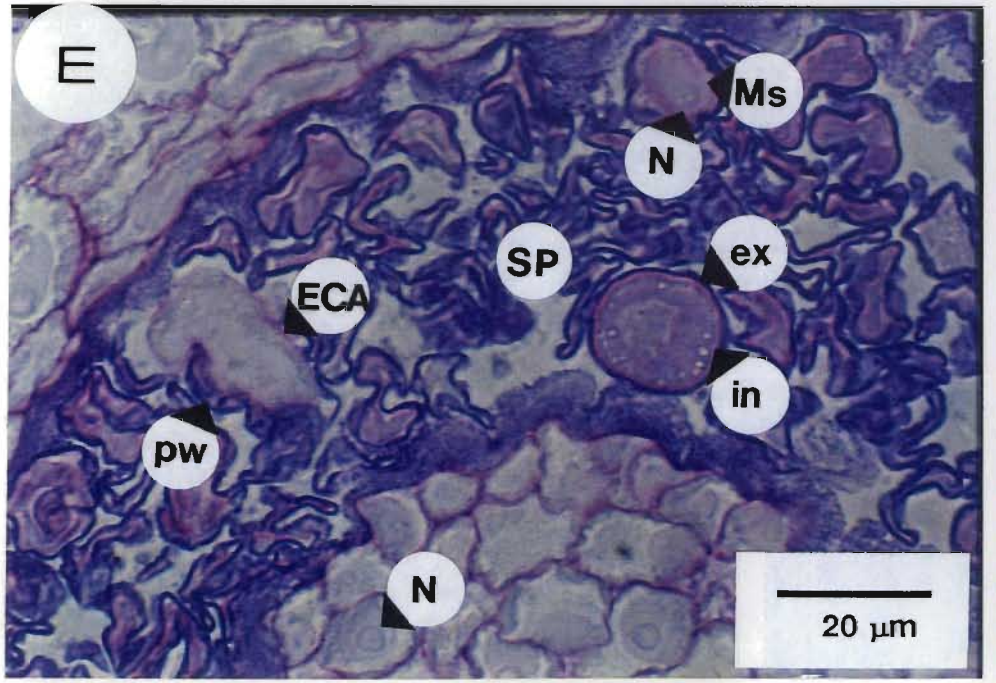
Figure 9.4 (ii) Cross-section of 87.2002/3 anthers fixed after a cold pre-treatment of 2 days and a culture period of 14 days on nutrient medium (Treatment 4) to indicate:

D. Degenerating tapetum, a multicellular microspore still enclosed within pollen sporoderm and many sterile pollen.
E. Mature, uninucleate pollen enclosed within pollen sporoderm (intine and exine) as well as embryogenic callus which is already released from the sporoderm were the exine ruptured.
F - G. A thin, degenerating tapetum, uninucleate endothecium cells and connective tissue. A mature microspore (pollen grain) surrounded by the thick exine of the pollen sporoderm, sterile pollen and a multicellular microspore enclosed within the sporoderm. The nuclei of the different cells contained in the multicellular microspore were divided by cell plates.

cp, cell plate(s); **ct**, connective tissue; **ECA**, embryogenic callus; **En**, endothecium; **ex**, exine; **in**, intine; **ml**, middle layer; **Ms**, microspore; **Mu**, multicellular microspore (young, developing embryoids); **n**, nucleolus; **N**, nucleus; **pw**, pollen sporoderm; **SP**, sterile pollen; **Tp**, tapetum.


D
SP
Mu
cp
N
n
Tp
20 µm


F
Tp
Ms
N
ct
ex
in
En
20 µm


E
Ms
N
SP
ex
ECA
in
pw
N
20 µm

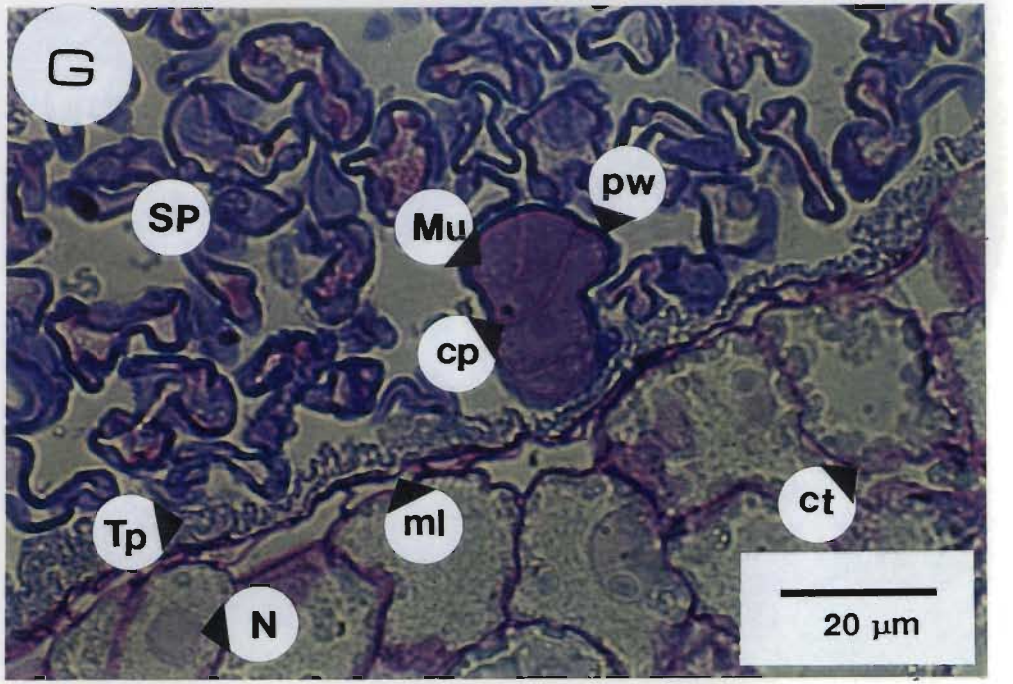

G
SP
Mu
pw
cp
Tp
ml
ct
N
20 µm

Fig. 9.4 (ii)

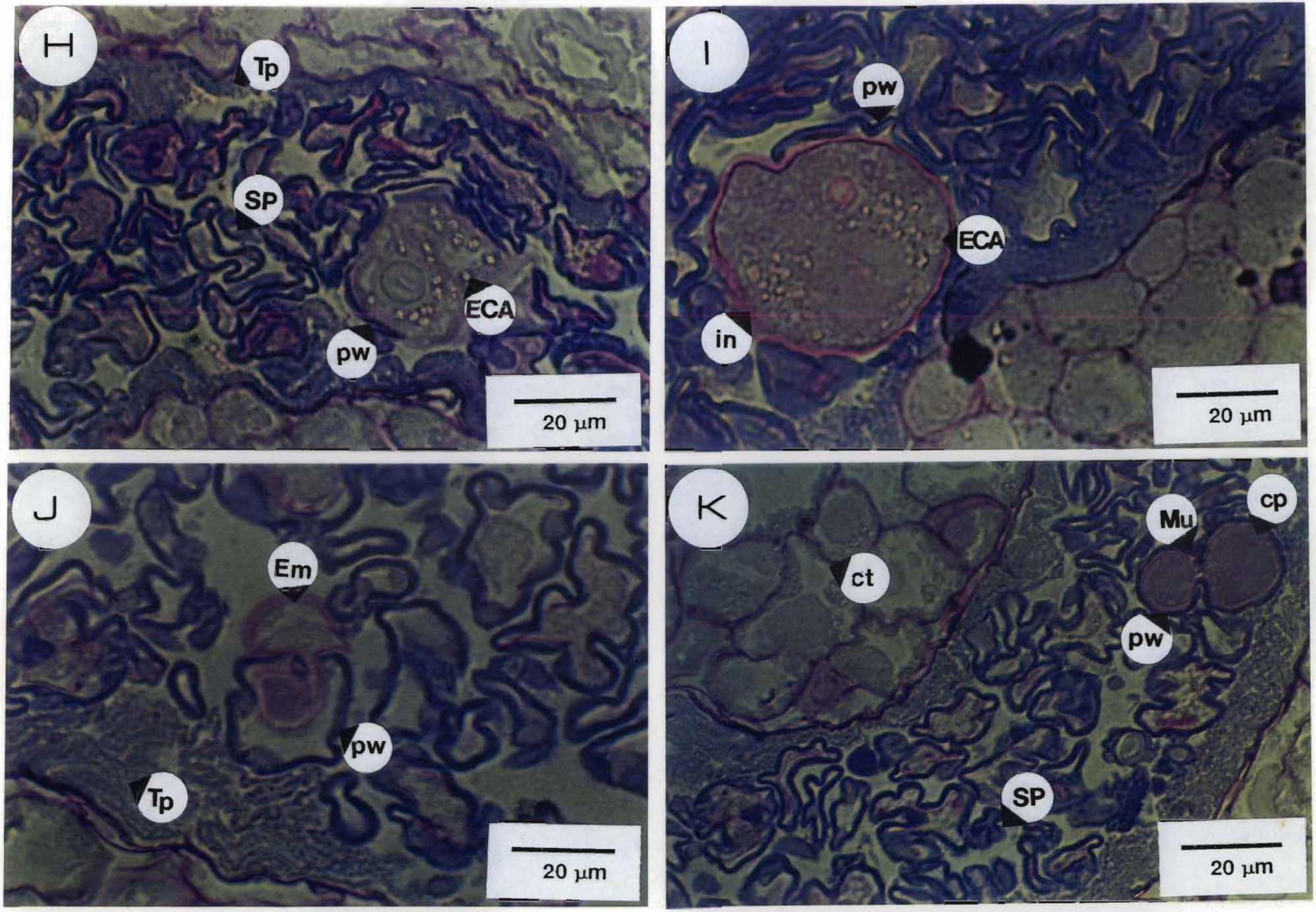
Figure 9.4 (iii) Cross-section of 87.2002/3 anthers fixed after a cold pre-treatment of 2 days and a culture period of 14 days on nutrient medium (Treatment 4) to indicate:

H. Remainder of the pollen sporoderm from which embryogenic callus have been released, sterile pollen and a thin degenerated tapetum.

I. Thick intine (pink) surrounding released embryogenic callus.

J - K. Sections from the same anther. Largely degenerated tapetum, sterile pollen, reieased embryoid (**J**) and remainder of pollen sporoderm, multicellular microspore enclosed within sporoderm.

cp, cell plate; **ct**, connective tissue; **ECA**, embryogenic callus; **Em**, embryoid; **in**, intine; **Mu**, multicellular microspores (young, developing embryoids); **pw**, pollen sporoderm; **SP**, sterile pollen; **Tp**, tapetum.


H
Tp
SP
ECA
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20 µm
I
pw
ECA
in
20 µm
J
Em
pw
Tp
20 µm
K
Mu
cp
ct
pw
SP
20 µm

F 4 (iii)

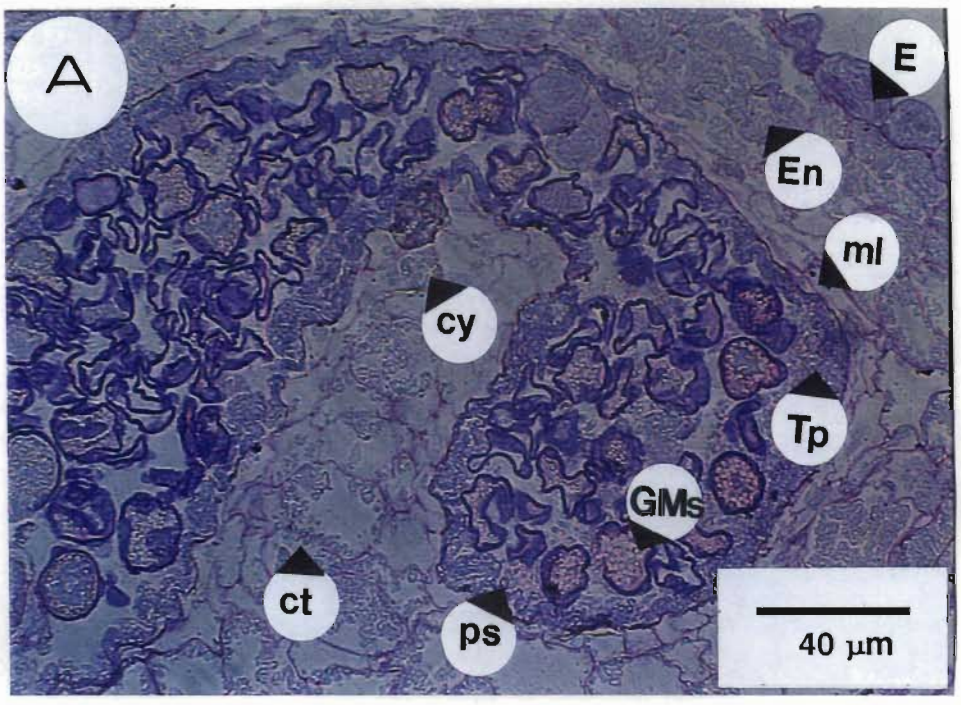
3.2.5 Treatment 5:

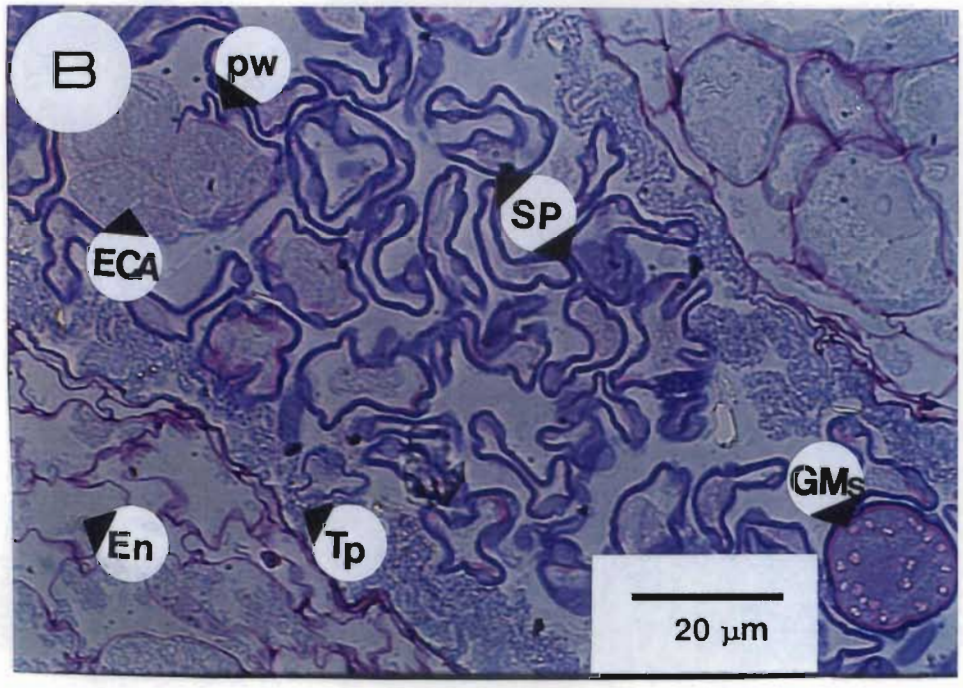
Both sterile microspores, mature granular microspores (GMs), multicellular microspores as well as embryogenic callus occurred at this stage (Figures 9.5 A - C). The granular microspores contained starch or fatty substances which was absorbed from the degenerating tapetum. This is also an indication of metabolic growth. The overall embryogenic development and tapetum degeneration did not appear more advanced compared to that of the previous treatment. However, at this stage - after a culture period of 21 days - the epidermis and the connective tissue started to degenerate more actively, inducing the pollen sacs to fuse for the first time. The cytoplasm of these surrounding epidermis and connective tissue, were plasmolyzed. Dehydration occurred to such an extent at this stage, that the opening of the anther wall at the stomium, for subsequent microspore/embryoid release onto the nutrient medium, was detected for the first time (Figure 9.5 D). Thus, although endothecium bands were clearly distinguishable in cultured anthers after **seven days**, anther-dehydration only reached a climax after 21 days, resulting in anther wall rupture. Thus, the period from the development of the U-shaped bands in the endothecium - as reported by FAHN (1974) - up to anther wall rupture and microspore liberation, took approximately two weeks. This agrees with the results of MAHESHWARI, RASHID and TYAGI (1982), whom observed that embryoids emerged from *Datura innoxia* anthers after a culture period of approximately 3 - 10 weeks.

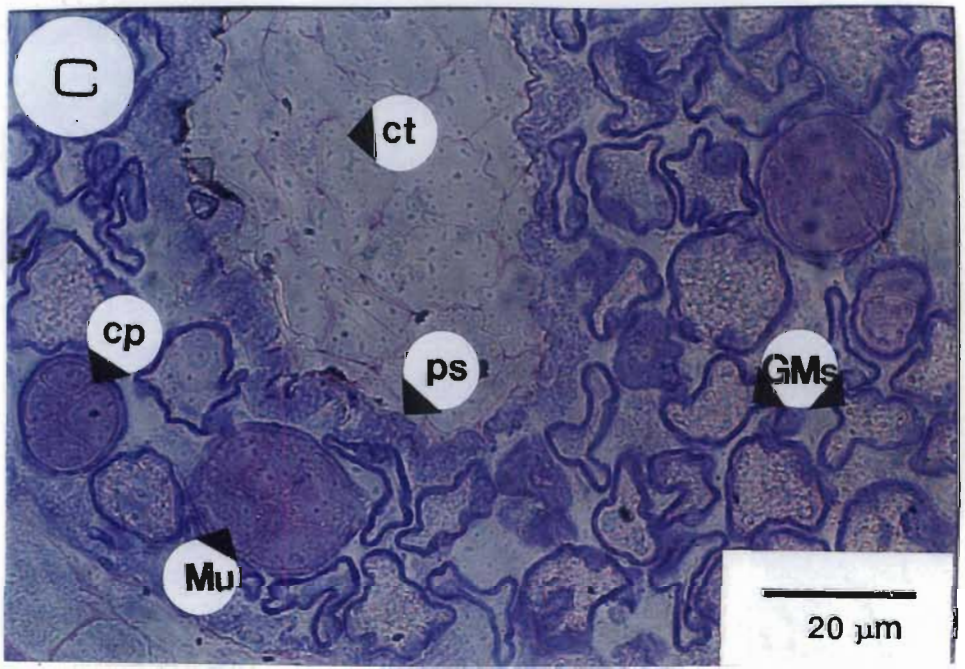
Figure 9.5 Cross-section of 87.2002/3 anthers fixed after a cold pre-treatment of 2 days and a culture period of 21 days on nutrient medium (Treatment 5) to indicate:

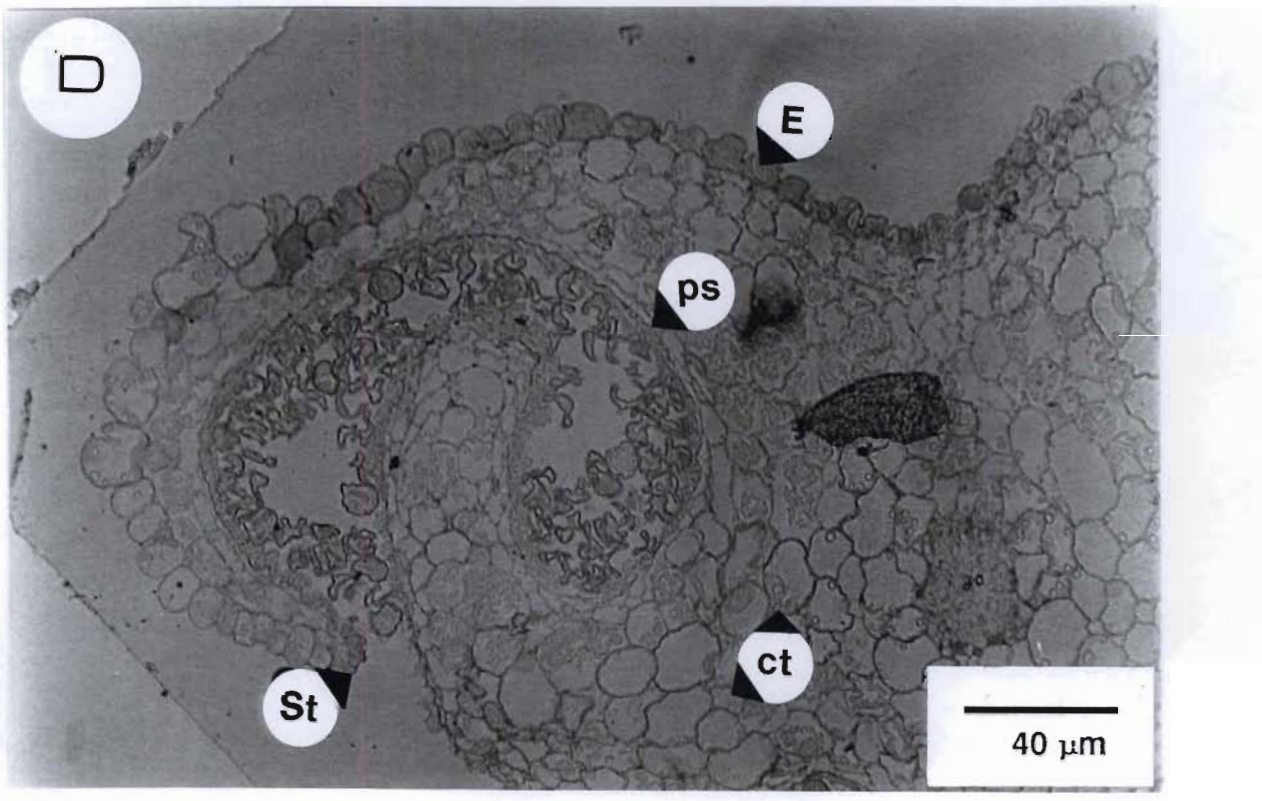
A. Part of a pollen sac containing many sterile pollen, largely degenerated tapetum, many granular microspores. Cytoplasm of endothecium and connective tissue clearly plasmolysed.
B. Embryogenic callus released from microspore with remaining pollen sporoderm, sterile microspores and a mature, granular microspore.
C. Pollen sac with mature, granular microspores and multicellular microspores still enclosed within pollen sporoderm and clearly developed cell plates.
D. Anther wall dehisced where stomium opened for microspore release from the pollen sacs. Epidermis and endothecium not showing any signs of degeneration.

cp, cell plate(s); **ct**, connective tissue; **cy**, cytoplasm; **E**, epidermis; **ECA**, embryogenic callus; **En**, endothecium; **GMs**, granular microspores; **ml**, middle layer; **Mu**, multicellular microspores (young, developing embryoids); **ps**, pollen sac; **pw**, pollen sporoderm; **SP**, sterile pollen; **St**, stomium; **Tp**, tapetum.


A
E
En
ml
cy
Tp
GMs
ct
ps
40 µm


B
pw
SP
ECA
GMs
En
Tp
20 µm


C
ct
cp
ps
GMs
Mu
20 µm


D
E
ps
ct
St
40 µm

3.2.6 Treatment 6:

Figure 9.6 A - B indicates a much higher concentration of enlarged microspores in relation to sterile pollen grains. In most of these enlarged microspores exine rupture occurred, releasing the young embryoids at the globular stage. In some of the other sections, high degrees of internal microbial contamination occurred (Figures C - D). In all anthers the pollen sacs were completely fused, while the surrounding epidermis, endothecium and connective tissue were in the process of degeneration. Only a few granular microspores were observed.

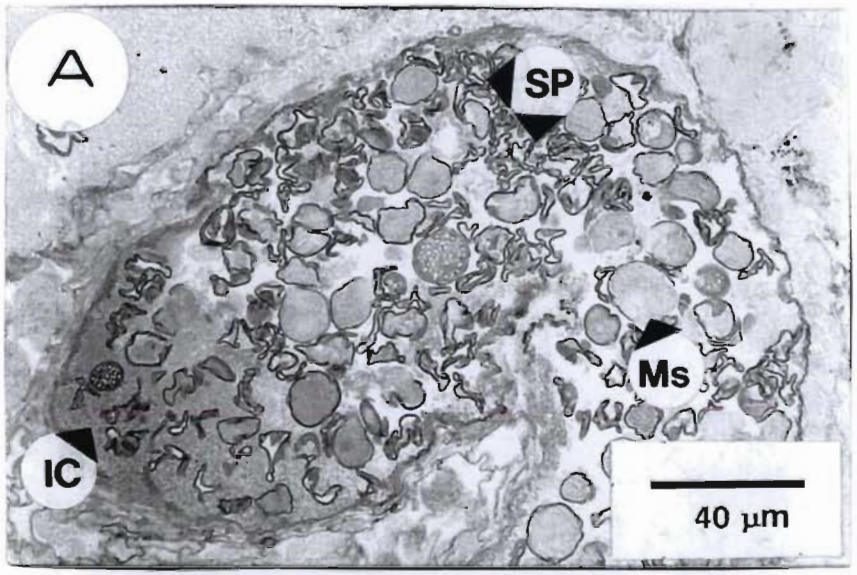
3.2.7 Treatment 7:

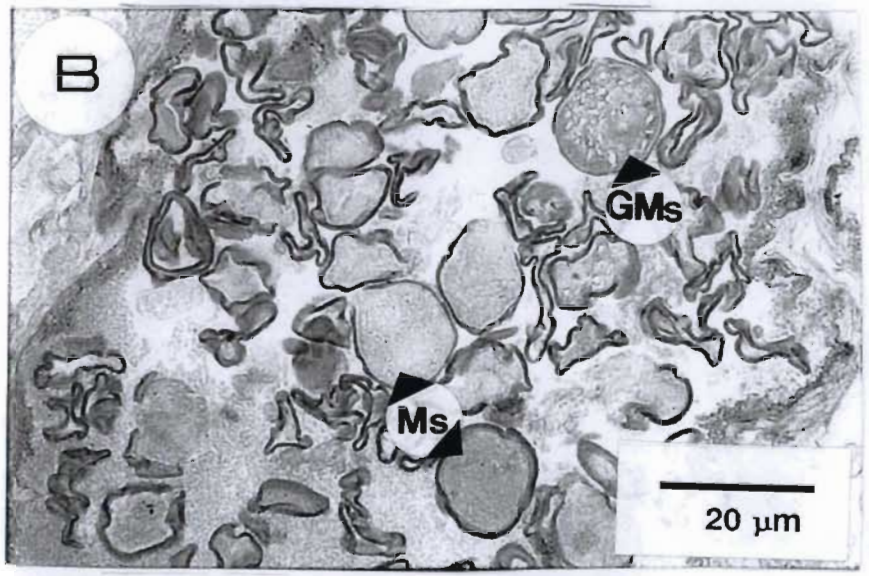
Figure 9.7 (A - C) shows that a high number of sterile microspores and granular microspores were present. The granular microspores contained starch and fatty substances, which were absorbed from the tapetum. Microspores had a "fluorescent" appearance. The tapetum was almost completely degenerated in most of the anthers studied. No embryogenic development was detected in these sections.

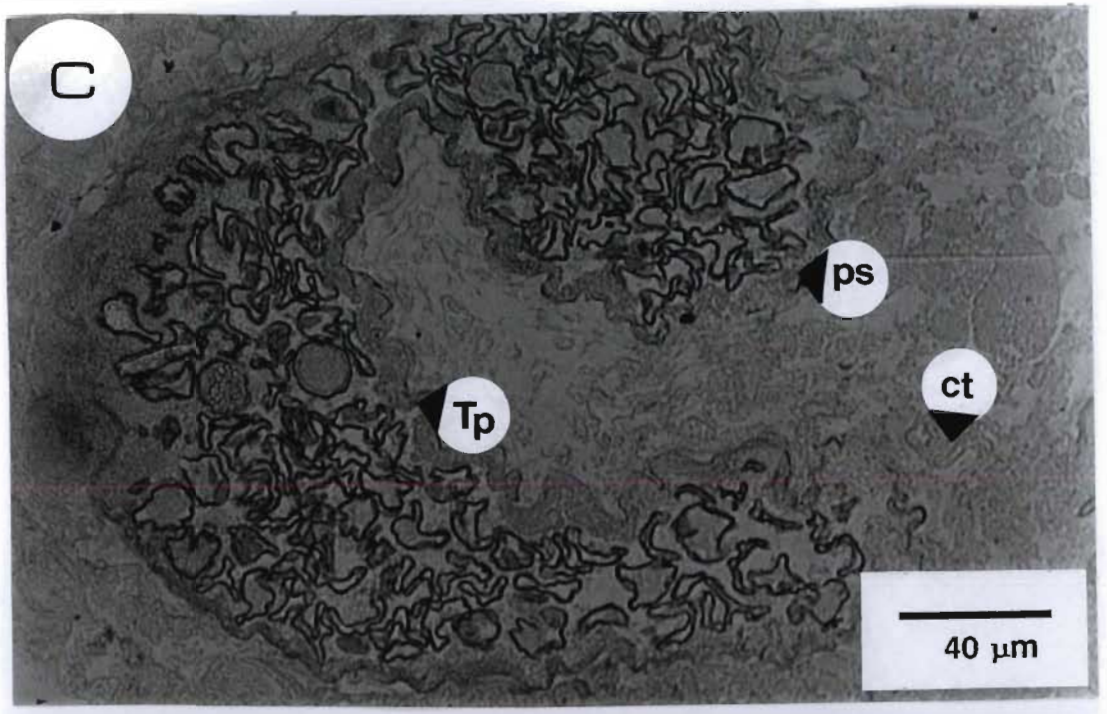
Figure 9.6 Cross-section of 87.2002/3 anthers fixed after a cold pre-treatment of 2 days and a culture period of 28 days on nutrient medium (Treatment 6) to indicate:

A - C. Part of a pollen sac containing many sterile pollen grains, largely degenerated tapetum, granular and enlarged microspores. Exine rupture occurred in many enlarged microspores. A high degree of internal microbial contamination at pollen sac ends can be seen and the connective tissue appeared degenerated.
D. Two connected pollen sacs with stomium still closed. Degenerating epidermis and endothecium and extensive internal microbial contamination at pollen sac ends.

ct, connective tissue; **E**, epidermis; **En**, endothecium; **GMs**, granular microspores; **IC**, internal microbial contamination; **Ms**, microspores; **ps**, pollen sac; **SP**, sterile pollen; **St**, stomium; **Tp**, tapetum.


A
SP
Ms
IC
40 µm


B
GMs
Ms
20 µm


C
ps
ct
Tp
40 µm

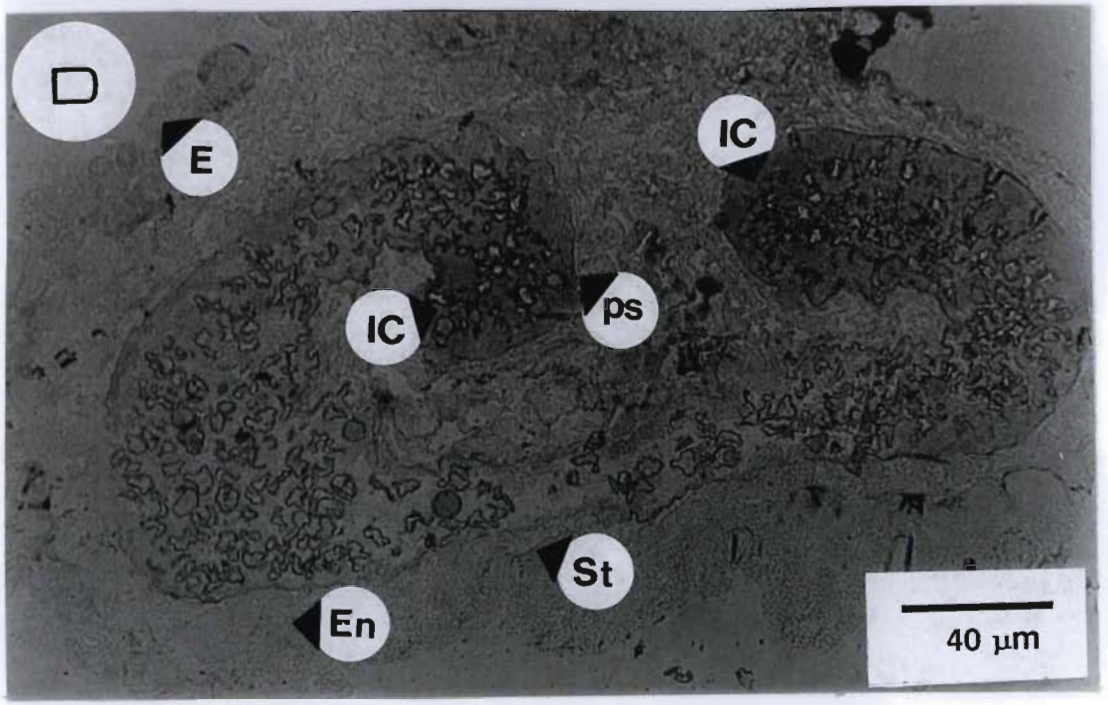
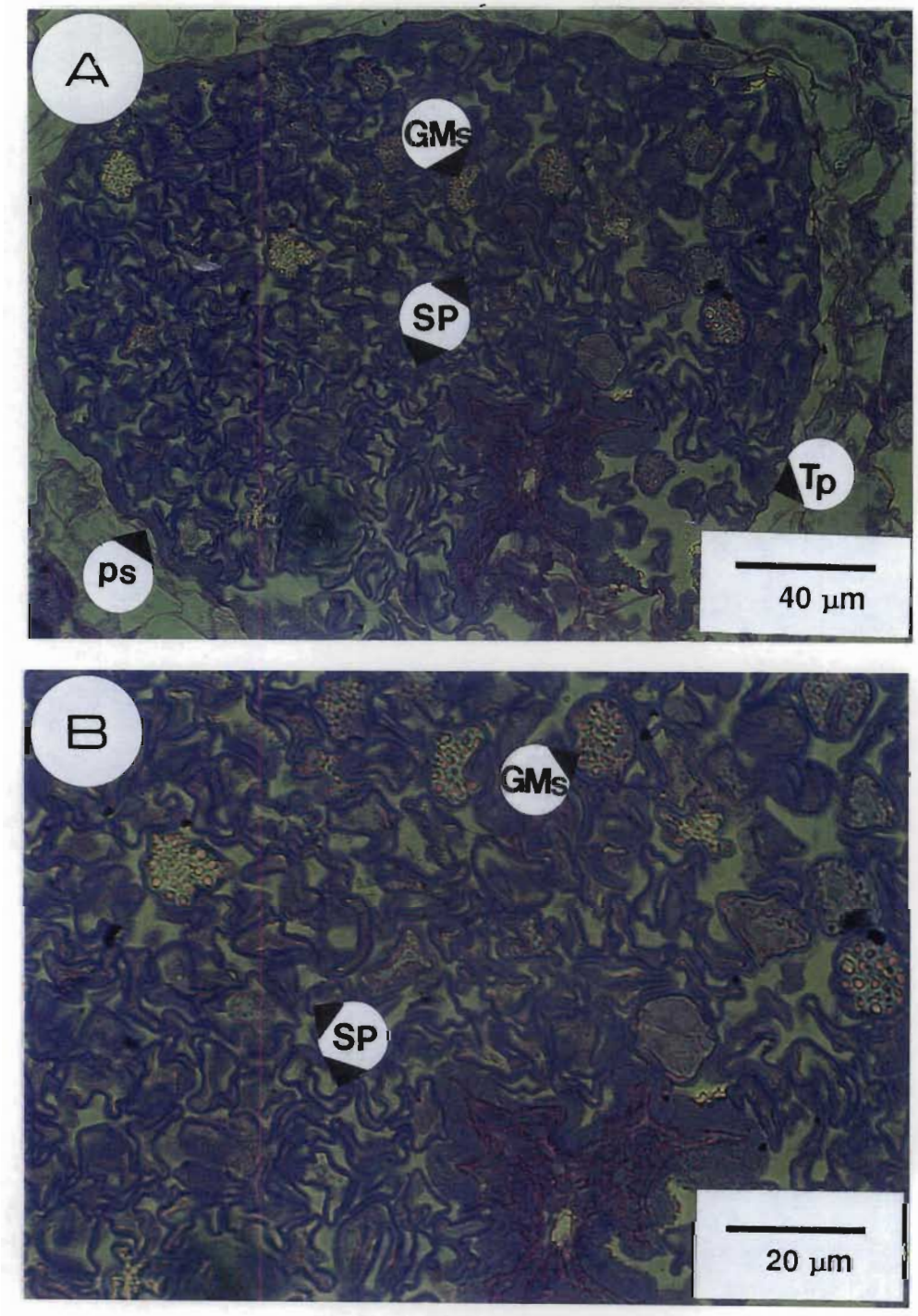

D
E
IC
IC
ps
St
En
40 µm

Figure 9.7 Cross-section of 87.2002/3 anthers fixed after a cold pre-treatment of 2 days and a culture period of 35 days on nutrient medium (Treatment 7) to indicate:

A - C. Pollen sac with large numbers of sterile microspores, granular microspores and almost completely degenerated tapetum.

GMs, granular microspores; **N**, nucleus; **ps**, pollen sac; **SP**, sterile pollen; **Tp**, tapetum.


A
GMs
SP
Tp
ps
40 µm
B
GMs
SP
20 µm
C
GMs
SP
N
20 µm

3.2.8 Treatment 8:

Embryogenic development was most prominent at this stage and could be seen in almost all the anthers. Huge embryoids, released from the pollen sporoderm, but still enclosed within the anther wall, were present (Figures 9.8 A - G). In Figures 9.8 A and B an embryoid, at the globular developmental stage and released from the pollen sporoderm, can be seen. A high amount of internal microbial contamination was also observed in some anthers (Figures B, F, G). In Figure 9.8 C, both a (released) globular embryoid and a multicellular microspore (still enclosed within pollen sporoderm) is visible. This again indicates that the process of microsporogenesis in potato anthers is not synchronized. Figures 9.8 D and E are consecutive sections of the same pollen sac (from the same anther). Figures 9.8 F and G are consecutive sections from another anther and of different pollen sacs. The cells of the endothecium became elongated. The tapetum was thin and degenerated.

Figure 9.8 (i) Cross-section of 87.2002/3 anthers fixed after a cold pre-treatment of 2 days and a culture period of 42 days on nutrient medium (Treatment 8) to indicate:

A. Pollen sac with sterile microspores, degenerating tapetum, a completely released globular embryoid, round cells of the connective tissue and elongated endothecium cell layer.
B. Globular embryoid completely released from the pollen sporoderm and a large degree of internal microbial contamination.
C. A globular embryoid as well as a multicellular microspore (developing embryoid enclosed within the thick exine of the pollen sporoderm) together in the same pollen sac.

ct, connective tissue; **Em**, embryoid; **En**, endothecium; **IC**, internal microbial contamination; **ml**, middle layer; **Mu**, multicellular microspore; **SP**, sterile pollen; **Tp**, tapetum.

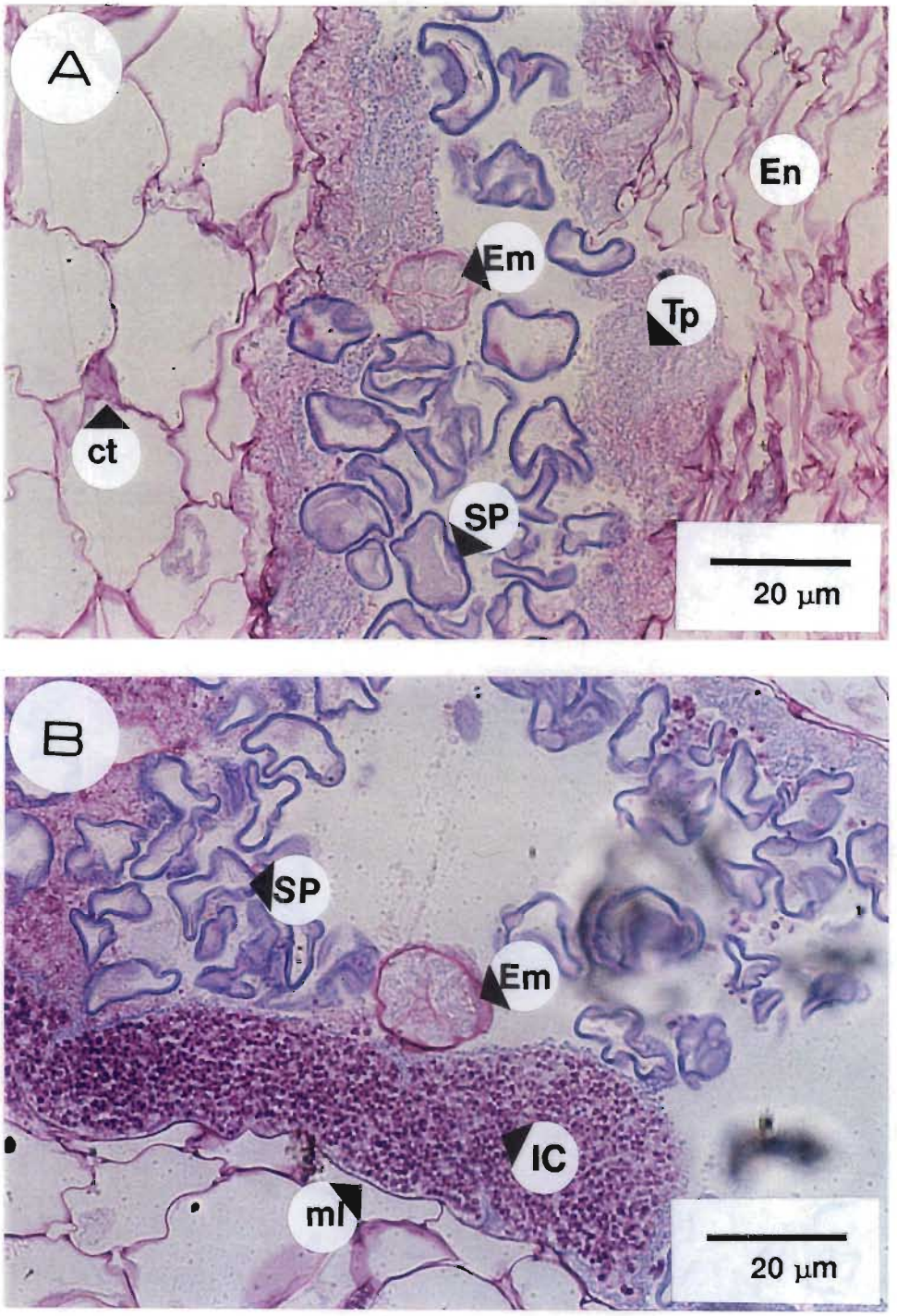
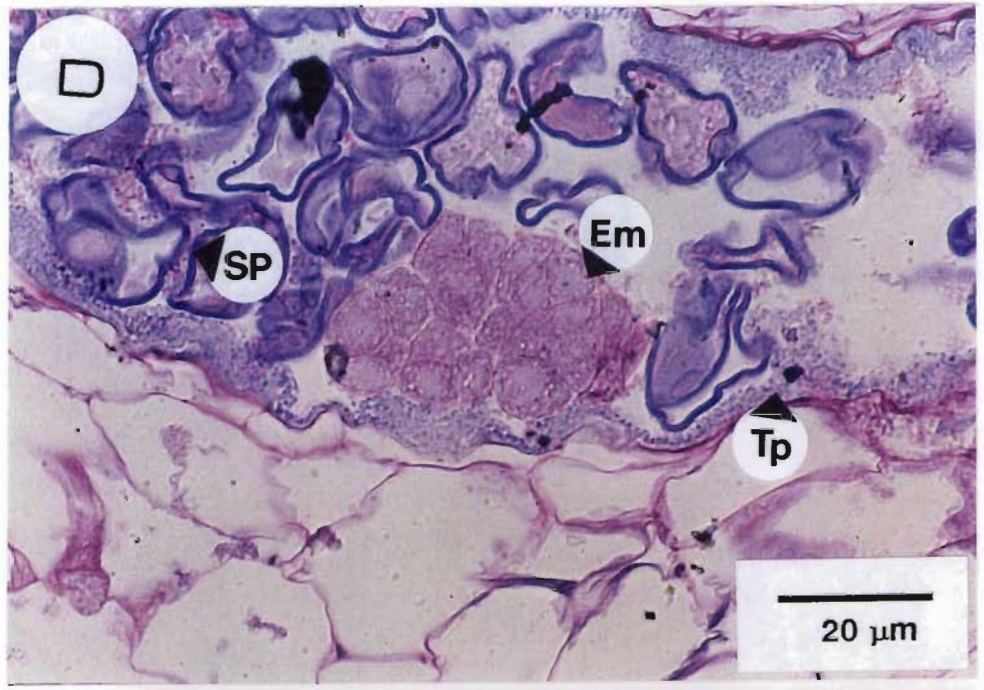

A
En
Em
Tp
ct
SP
20 µm
B
SP
Em
IC
ml
20 µm
C
En
Em
Tp
Mu
20 µm

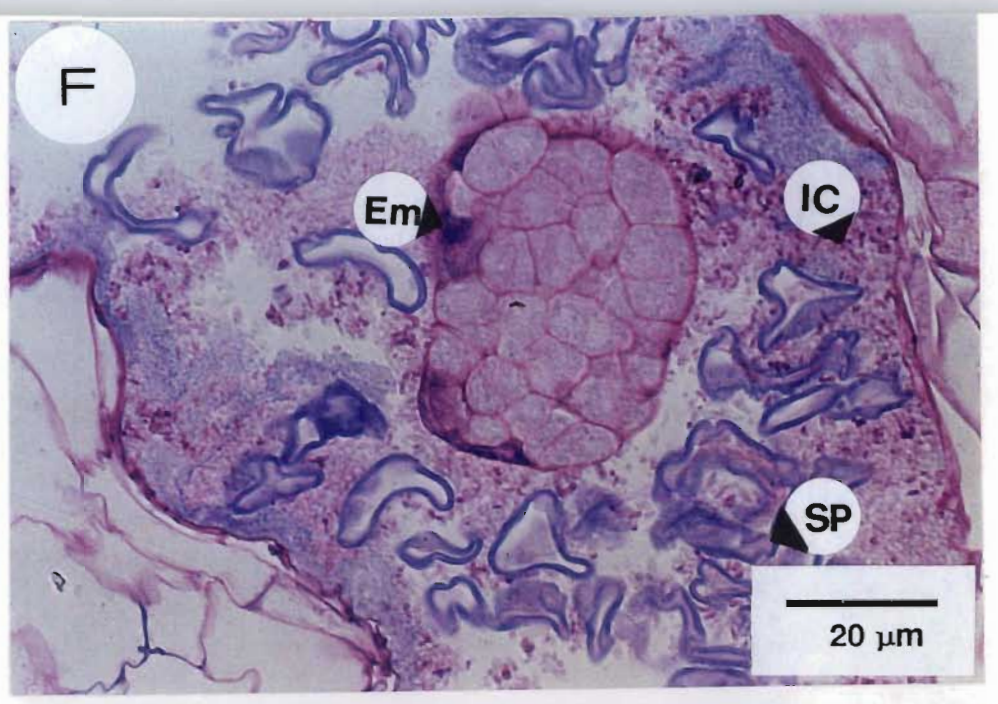
Figure 9.8 (ii) Cross-section of 87.2002/3 anthers fixed after a cold pre-treatment of 2 days and a culture period of 42 days on nutrient medium (Treatment 8) to indicate:

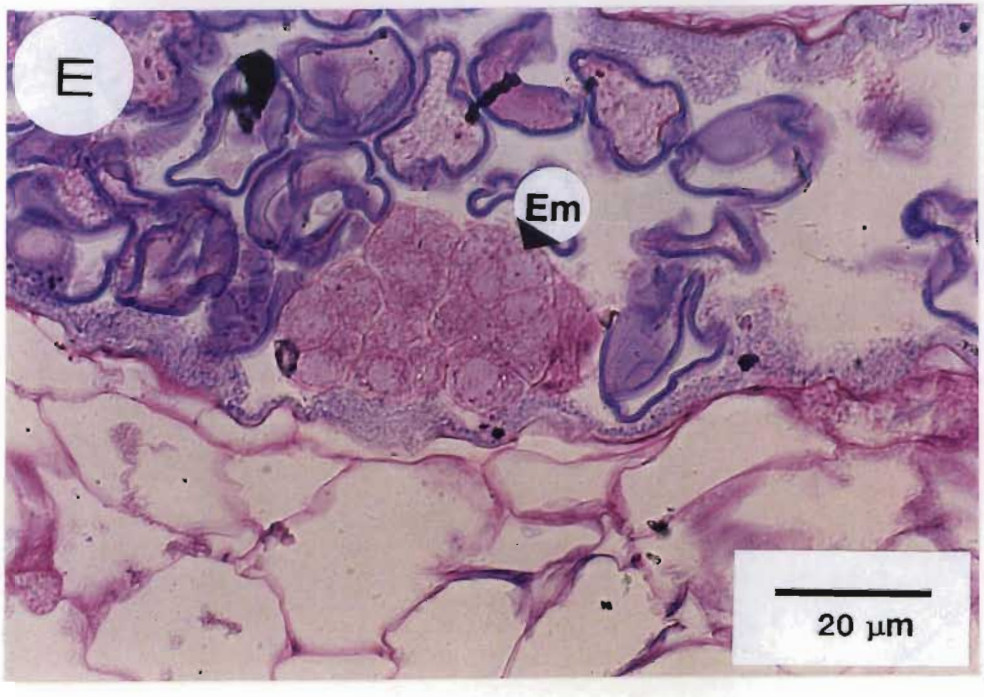
D - E. Consecutive sections of the same anther: Thin degenerated tapetum, sterile pollen and large embryoid development.

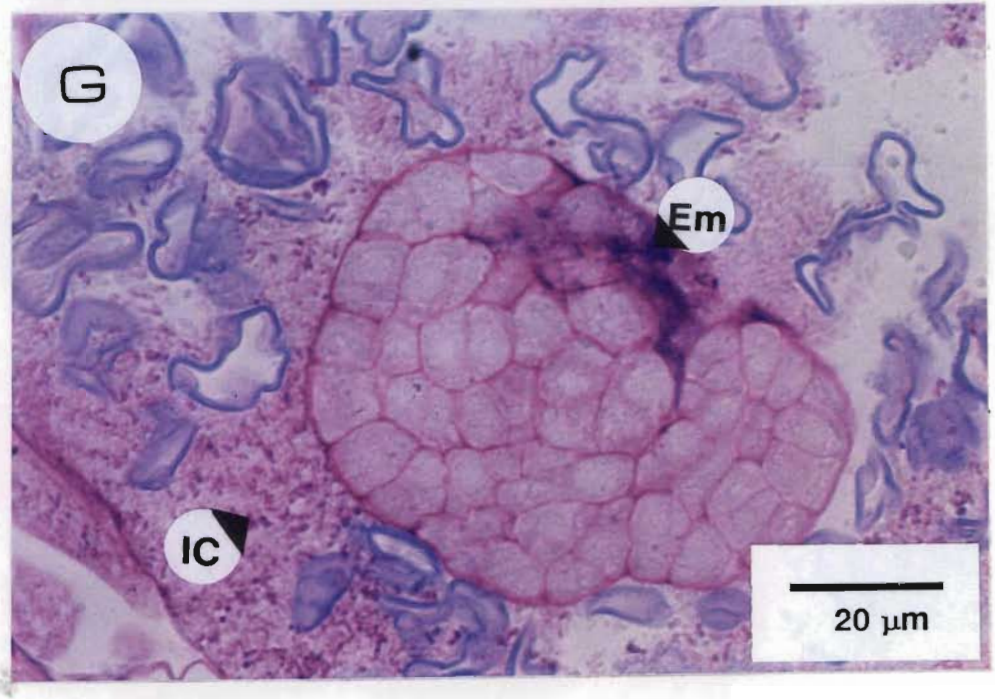
F - G. Consecutive sections of one anther but of different pollen sacs: Sterile pollen, internal microbial contamination and a large embryoid starting to differentiate into a plantlet.

Em, embryoid; **IC**, internal microbial contamination; **SP**, sterile pollen; **Tp**, tapetum.


D
SP
Em
Tp
20 µm


F
Em
IC
SP
20 µm


E
Em
20 µm


G
Em
IC
20 µm

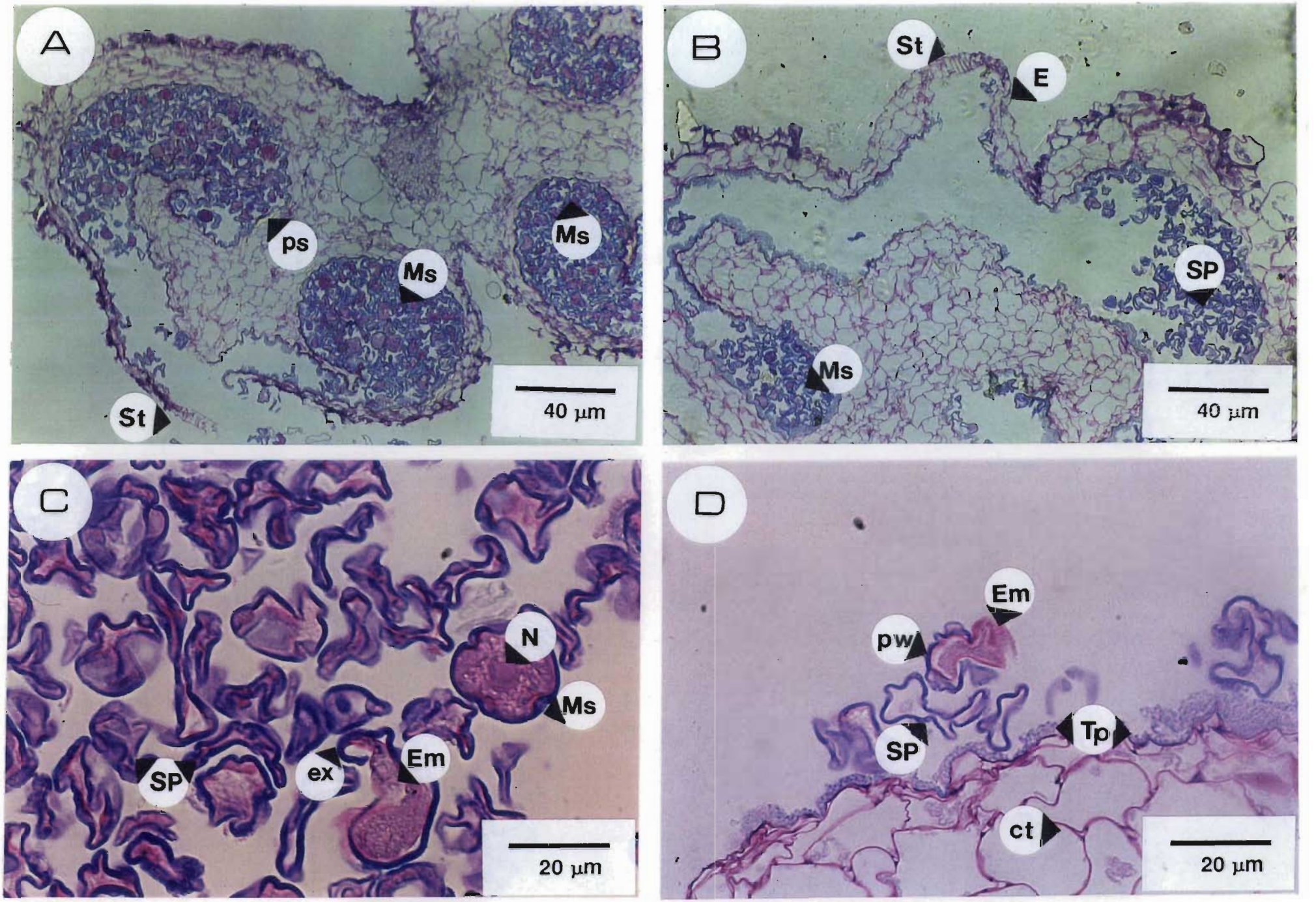
3.2.9 Treatment 9:

Anthers of this treatment were cultured initially for 28 days on the nutrient medium after which they were transferred to fresh medium. After a second culture period of 14 days on the fresh nutrient medium, the anthers were selected and fixed for microscopic studies. Thus, these anthers were also cultured for a period of 42 days before fixation - similar to that in Treatment 8 - with the exception that anthers of Treatment 8 remained for the duration of the culture period on the same nutrient medium and were not transferred to fresh medium after four weeks. Figures 9.9A and B indicate that in some cases the stomium had already opened while it remained closed in others. Where the stomium was still closed, the region where dehiscence should occur was covered by a single epidermal cell layer. The pollen sacs were completely fused. The epidermis and endothecium were almost completely degenerated and the connective tissue also showed signs of disintegration. The tapetum had degenerated completely at some places and was very thin where it still occurred. Microspore release from the anther is evident in Figure 9.9 A where the stomium had broken. A large number of sterile microspores were present as well as a mature, uninucleate microspore which did not develop any further (Figure 9.9C). Embryoid formation was also visible, with embryoid release occurring where the exine ruptured (Figure D).

Figure 9.9 Cross-section of 87.2002/3 anthers which received a cold pre-treatment for 2 days, were cultured for 28 days on nutrient medium, transplanted to fresh medium and fixed after remaining for another 14 days on this fresh medium (Treatment 9) to indicate:

A. Part of a complete anther which shows the degenerated epidermis and endothecium, the remainder of the stomium after opening and two connected pollen sacs containing mature sterile pollen grains. These were mainly concentrated at pollen sac ends. Microspores were released from the anther where stomium ruptured.
B. Two connected pollen sacs surrounded only by a single epidermal layer in the region where the stomium should rupture.
C. Sterile pollen and a few mature, uninucleate microspores enclosed within the pollen sporoderm. In some microspores, the exine started to rupture.
D. An almost completely degenerated tapetum, sterile pollen grains and an embryoid released from pollen where exine had ruptured.

ct, connective tissue; **E**, epidermis; **Em**, embryoid; **ex**, exine; **Ms**, microspore; **N**, nucleus; **ps**, pollen sac; **pw**, pollen sporoderm; **SP**, sterile pollen grains; **St**, stomium; **Tp**, tapetum; **UN**, uninucleate microspores.


A
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C
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Ms
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20 µm
D
Em
pw
SP
Tp
ct
20 µm

3.2.10 Treatment 10:

Anthers of this treatment were also transferred after 28 days to fresh medium, as in Treatment 9, but were fixed after an culture period of 28 days. In Figures 9.10 A - B it can be seen that the stomium as still closed in some of the anthers studied. In others, the stomium had already ruptured. The degeneration of the epidermis, endothecium, connective tissue and the tapetum had occurred to a great extent after such a long culture period and correlated with that in Treatment 9. Embryoid development and release from the pollen sporoderm occurred (Figures C, D). A number of granular microspores were also observed. The pollen sporoderm appeared thick.

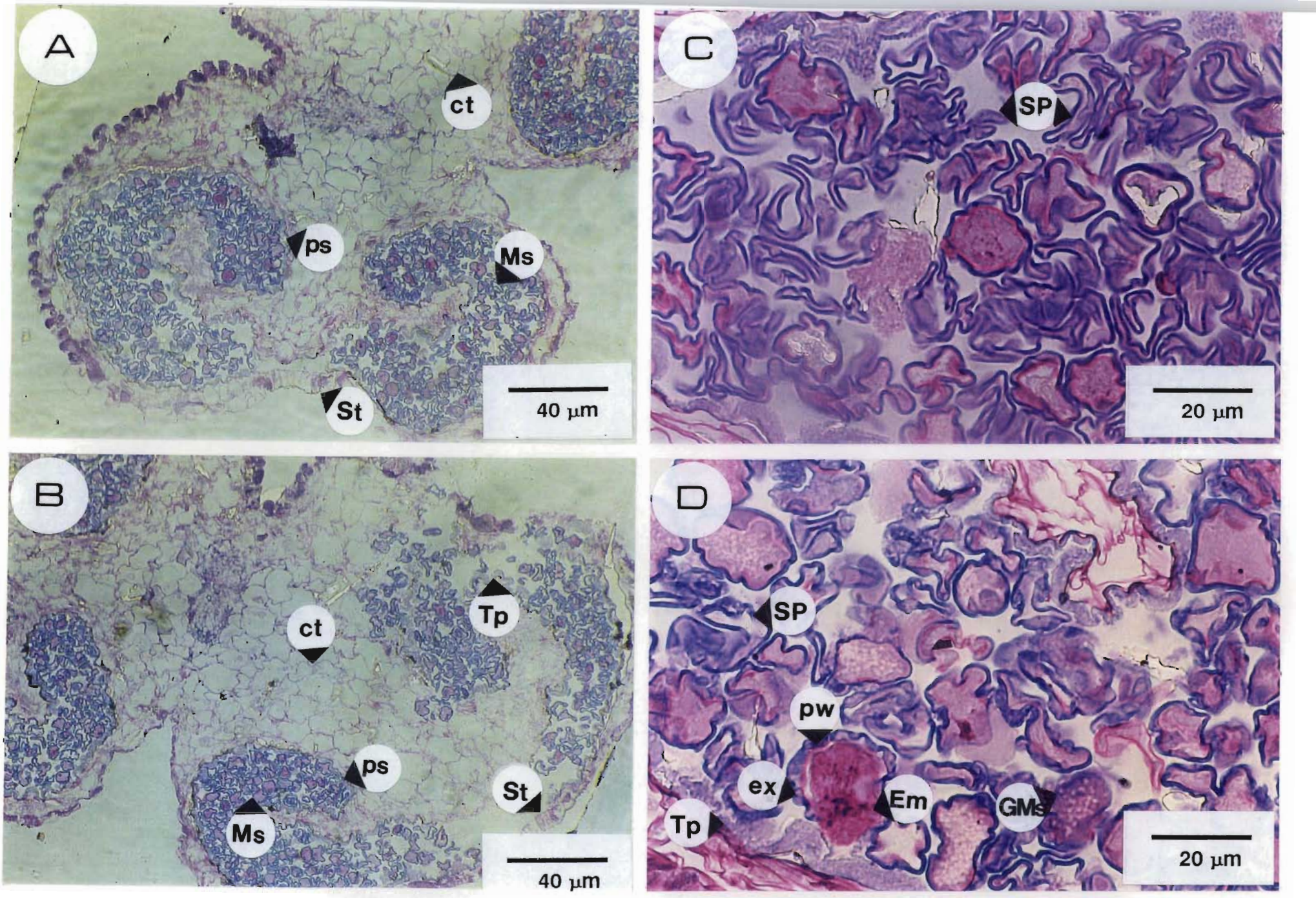
In both Treatments 9 and 10 less embryoid development was observed compared to the other treatments. Larger numbers of sterile and/or mature microspores were observed as well as a few at the point of embryoid release. Apparently the microspores did not develop further after the anthers were transferred to fresh nutrient medium. This could indicate that only in those microspores amenable to androgenetic response, embryoid production would be induced early in (within the first week of) the culture period. Thus, a transfer to fresh medium with new growth regulators would not necessarily increase the number of responsive anthers.

Figure 9.10 Cross-section of 87.2002/3 anthers which received a cold pre-treatment of 2 days, were cultured for 28 days on nutrient medium, transplanted to fresh medium and fixed after remaining for another 28 days on this fresh medium (Treatment 10) to indicate:

A - B. Two opposite sides of the same anther showing the two connected pollen sacs of each lobe as well as the stomium which is still closed in both anther lobes. Epidermis, endothecium and tapetum are degenerated.

C - D. Sections from the same anther showing granular- and sterile microspores as well as the remainder of the pollen sporoderm where the exine ruptured for embryoid release. Exine of pollen sporoderm appear thick.

ct, connective tissue; **E**, epidermis; **ex**, exine; Em, embryoid; **GMs**, granular microspore; **ps**, pollen sac; **pw**, pollen sporoderm; **SP**, sterile pollen; **St**, stomium; **Tp**, tapetum.


A
ct
ps
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40 µm
C
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B
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St
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40 µm
D
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pw
ex
Em
GMs
Tp
20 µm

3.3 Interpretation of microtome sections of the anthers of tetraploid cultivar Atzimba

3.3.1 Treatment 1:

Material of this treatment was fixed immediately after harvest, without a cold pre-treatment or a culture period on nutrient medium. Most microspores were at the uninucleate stage of development. The late binucleate stage of microspore development was also distinguished due to the prominent nucleoli present in a number of microspores (Figures 10.1 B). A limited number of bicellular microspores also occurred. Due to the occurrence of both the uni-, binucleate and bicellular developmental stages, it can be concluded that the microspores were in the process of mitosis. The microspores were slightly deformed and less round in shape compared to that of breeding line 87.2002/3 (Treatment 1). The tapetum was well developed and thick, suggesting an active nutritive function. Uninucleate connective tissue surrounded the pollen sacs. In all microtome sections studied, the four pollen sacs were not yet fused and the stomium was visible, but still closed. In Figures 10.1 A and 10.1 D the elongated stomium cells between the two pollen sacs - in the region where dihiscion would occur - were discernable.

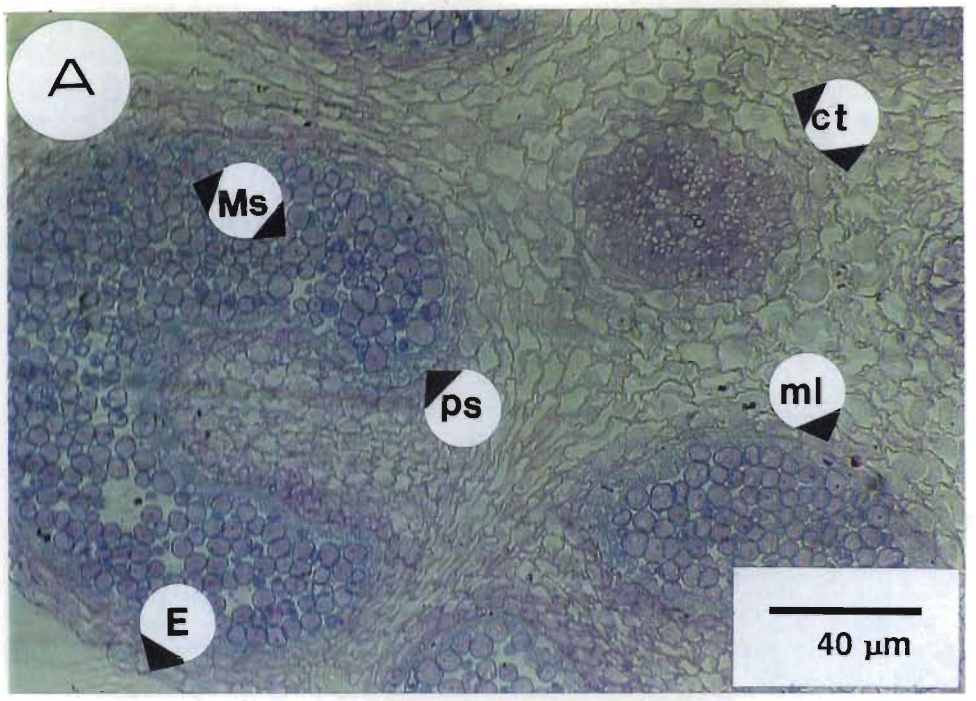
Figure 10.1 Cross-section of Atzimba anthers fixed immediately after harvest, without a cold pre-treatment and without culturing on nutrient medium (Treatment 1) to indicate:

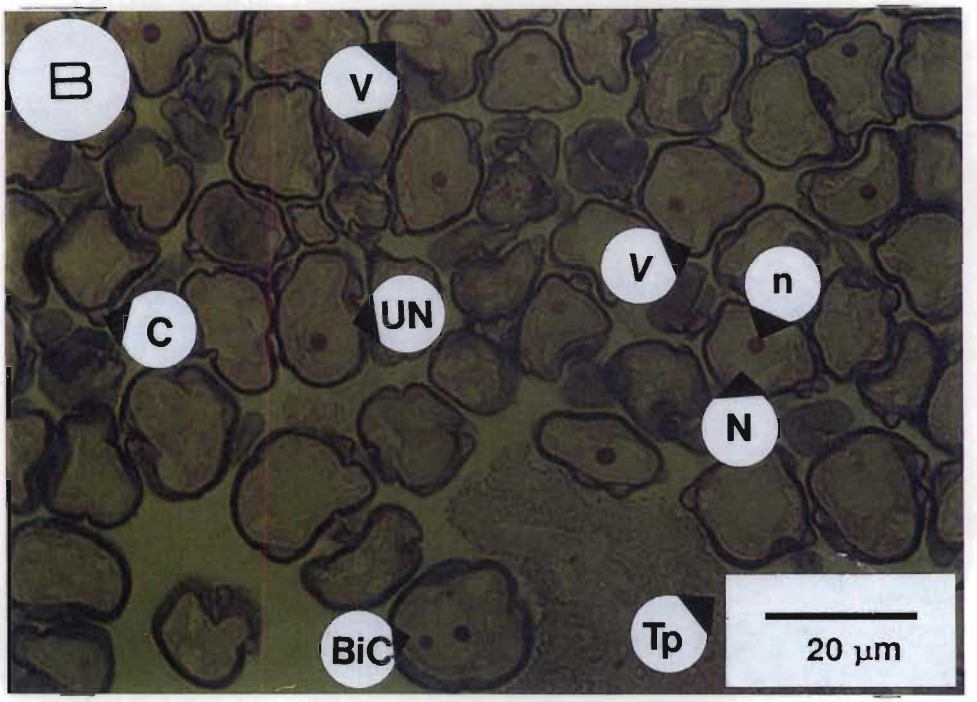
A. The two pollen sacs present in one anther lobe containing microspores and showing the epidermis, endothecium and middle layer as well as the connective tissue between the pollen sacs. The tapetum appear thick and well developed.

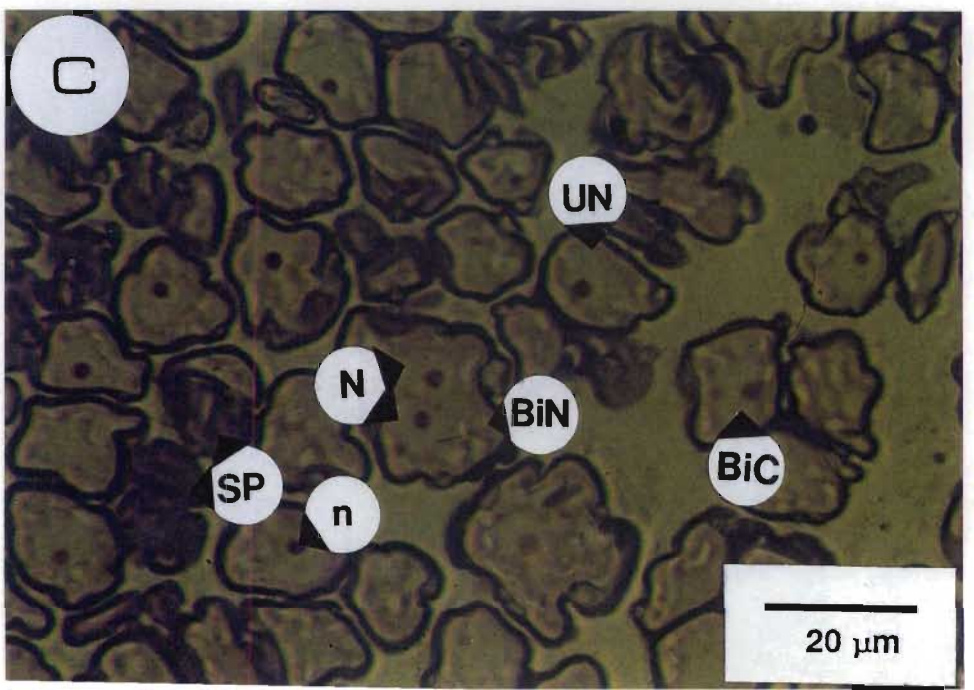
B - C. Microspores at the uninucleate and binucleate stage of development as well as sterile pollen present in the same pollen sac. A limited number of bicellular microspores were also distinguished (B). Some of the colpi of the tricolpate microspores were also visible. All microspores were semi-round or slightly deformed in shape, surrounded by thick pollen sporoderms (blue). A few microspores contained vacuoles.

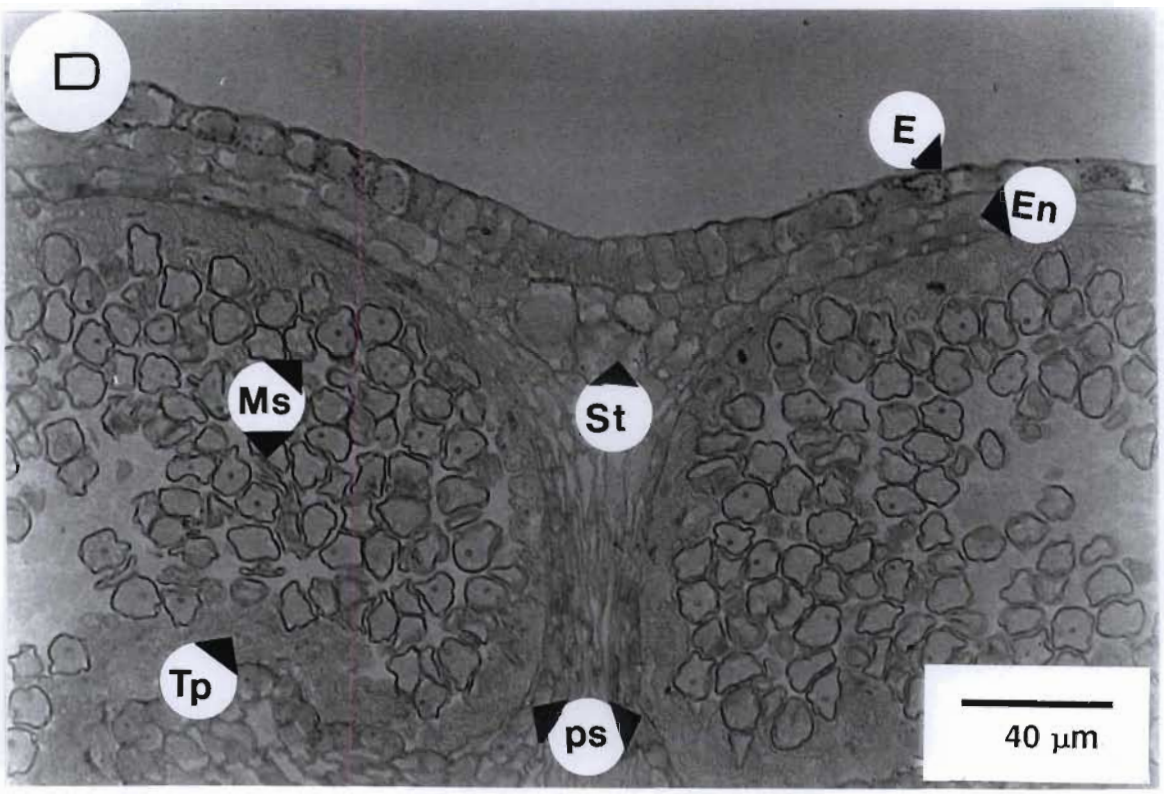
D. Elongated cells visible between pollen sacs in the region where dehiscence will occur.

BiC, bicellular microspore; **BiN**, binucleate microspores; **C**, colpus(i); **ct**, connective tissue; **E**, epidermis; **En**, endothecium; **ml**, middle layer; **Ms**, microspores; **N**, nucleus; **ps**, pollen sac; **SP**, sterile pollen; **Tp**, tapetum; **Tr**, tricolpate microspores; **UN**, uninucleate microspores; **V**, vacuole.


A
ct
Ms
ps
ml
E
40 µm


B
V
V
n
C
UN
N
BiC
Tp
20 µm


C
UN
N
BiN
BiC
SP
n
20 µm


D
E
En
Ms
St
Tp
ps
40 µm

3.3.2 Treatment 2:

The anthers of this treatment all received a cold pre-treatment of two days before fixation. Most of the microspores contained in these anthers were more round in shape compared to those in Treatment 1. This differed from the observations made for breeding line 87.2002/3 (Treatment 2) where microspores appeared less round in shape after the cold pre-treatment. Uninucleate, binucleate as well as a few sterile microspores were present (Figures 10.2 A - B). The late binucleate stage of microspore development was also distinguished. A large number of granular microspores were detected, suggesting the possibility of starch depositioning. Starch depositioning only became prominent in the microspores of line 87.2002/3 after a culture period of 21 days. This could imply that the anthers of Atzimba were selected too late, as microspore development and embryoid production *in vitro* cannot be induced after starch depositioning had already taken place. The occurrence of the different microspore developmental stages again confirmed that the microspores were in the process of active mitosis as in Treatment 1. Some of the microspores contained one or more vacuoles (Figures 10.2 B).

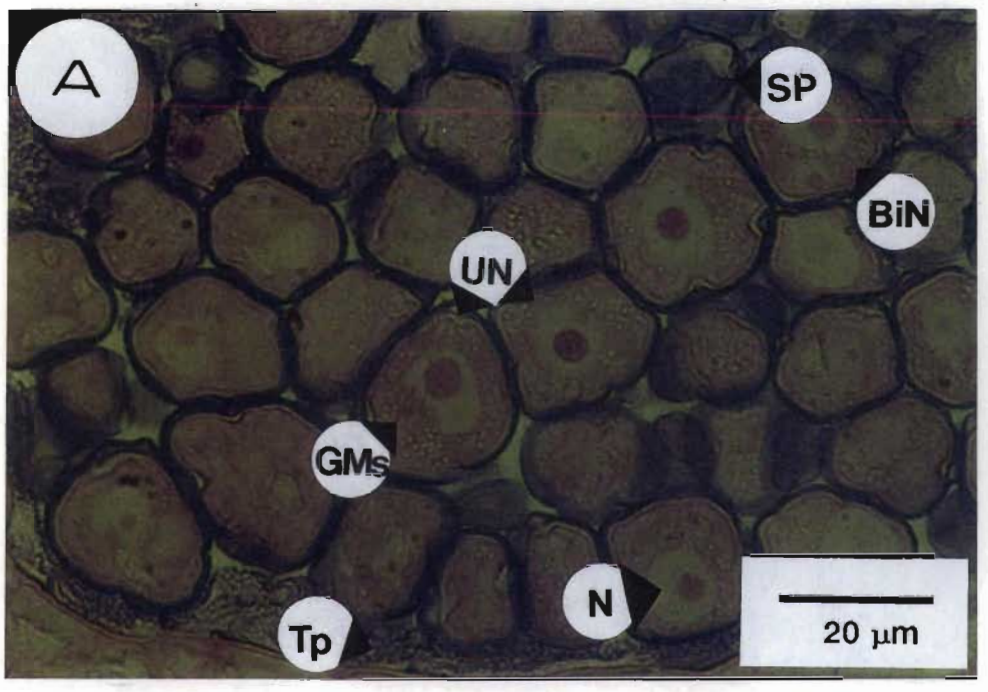
3.3.3 Treatment 7:

These results agreed to a great extent with those for breeding line 87.2002/3 (Treatment 7). Figures 10.3 A - E shows the occurrence of many sterile microspores, surrounded by a relatively thick tapetum. The pollen sacs were either fused and the anther wall appeared open where the stomium dehisced or the pollen sacs were not yet connected and the stomium appeared closed. The cytoplasm was plasmolyzed and the endothecium and connective tissues were degenerated. No embryogenic development was detected in any sections of this treatment.

Figure 10.2 Cross-section of Atzimba anthers fixed after a cold pre-treatment of 2 days but without culturing on nutrient medium (Treatment 2) to indicate:

A - B. Microspores were all round in shape after the cold pre-treatment, with thick pollen sporoderms. Both uninucleate, binucleate and vacuolated microspores could be distinguished. A large number of granulated microspores and only a few sterile pollen grains were visible. The tapetum was much thinner compared to that of breeding line 87.2002/3.

BiN, binucleate microspores; **GMs**, granular microspores; **Ms**, microspores; **N**, nucleus; **SP**, sterile pollen; **Tp**, tapetum; **UN**, uninucleate microspores; **V**, vacuole.


A
SP
BiN
UN
GMs
N
Tp
20 µm

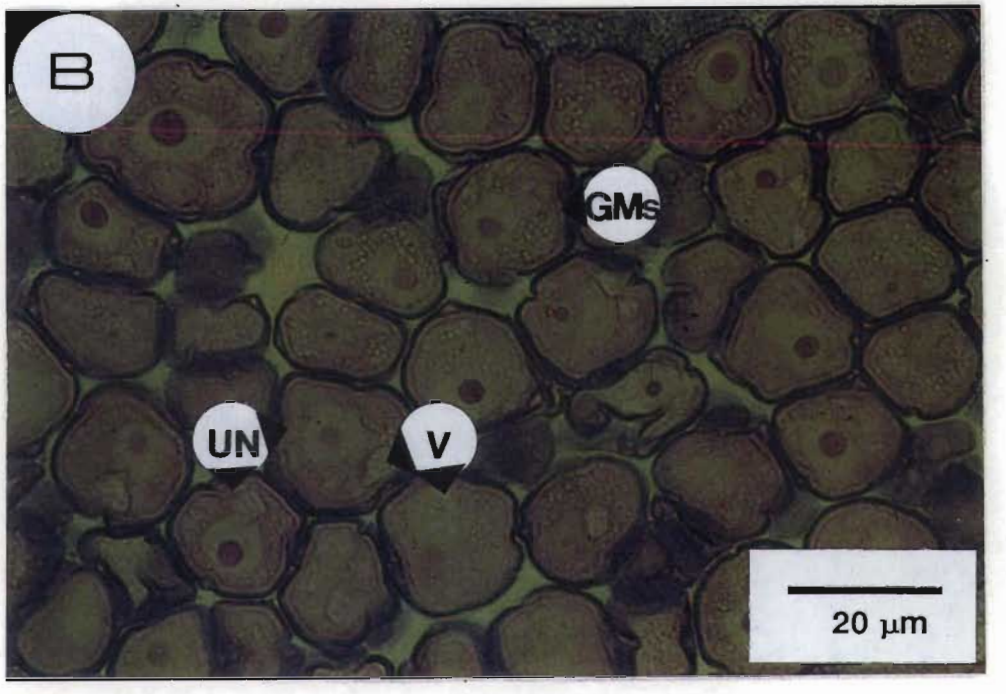

B
GMs
UN
V
20 µm

Figure 10.3 Cross-section of Atzimba anthers fixed after a cold pre-treatment of 2 days and a culture period of 35 days on nutrient medium (Treatment 7) to indicate:

A. Two pollen sacs in one anther lobe containing a large number of sterile pollen. The anther is enclosed by a degenerating epidermis, while the cytoplasm of the connective tissue appears plasmolysed. The stomium is quite prominent where the two pollen sacs have not yet connected.

B. Two connected pollen sacs with tapetum bridge/connection still obvious between them. Degenerated epidermis and endothecium.

C -D. Much thicker tapetum, a uninucleate microspore and many sterile pollen grains.

E. Anther wall in the process to dehisce at stomium opening.

ct, connective tissue; **cy**, cytoplasm; **E**, epidermis; **En**, endothecium; **IC**, internal microbial contamination; **ps**, pollen sac; **SP**, sterile pollen; **St**, stomium; **Tp**, tapetum; **TB**, tapetum bridge; **UN**, uninucleate microspore.

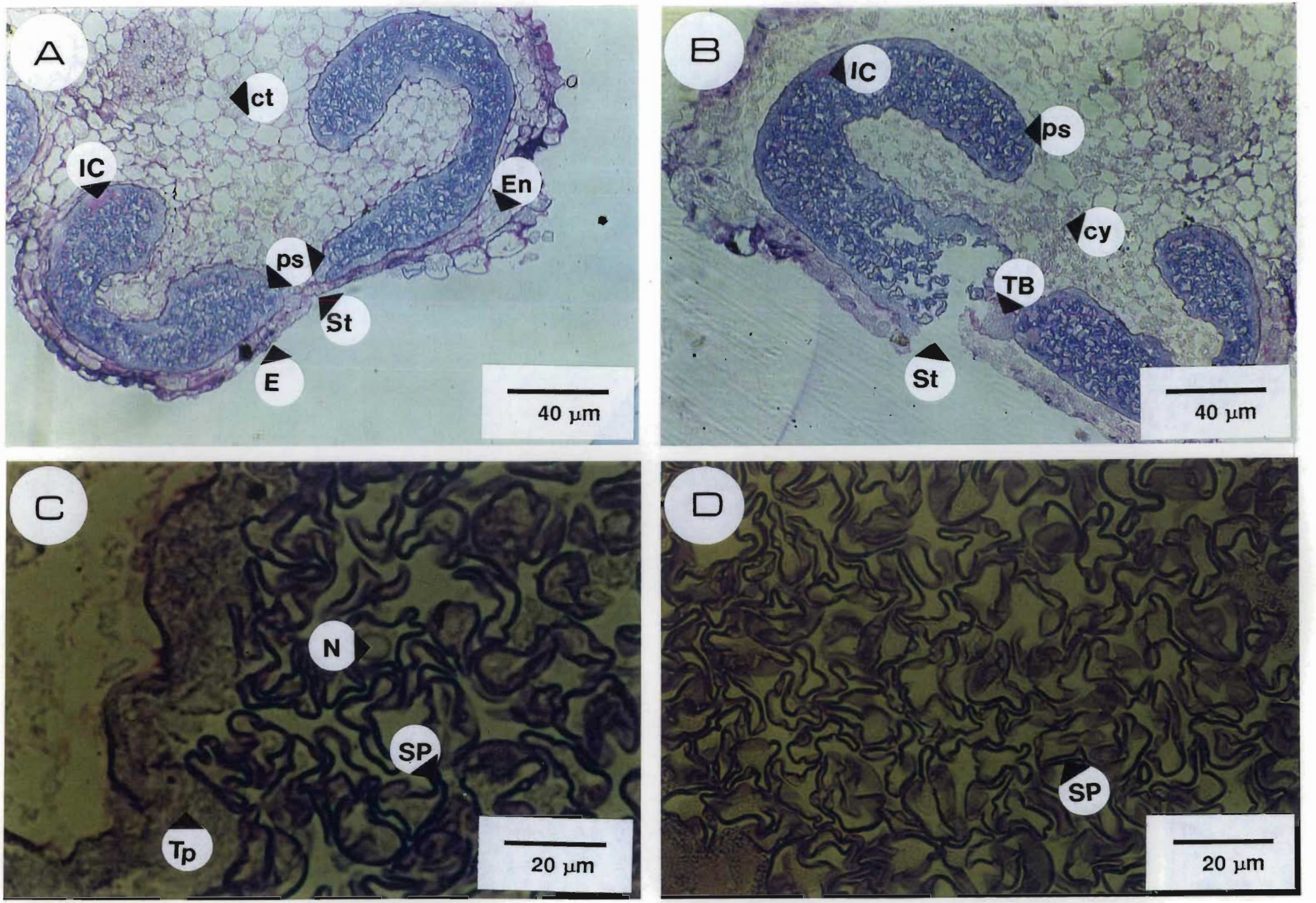
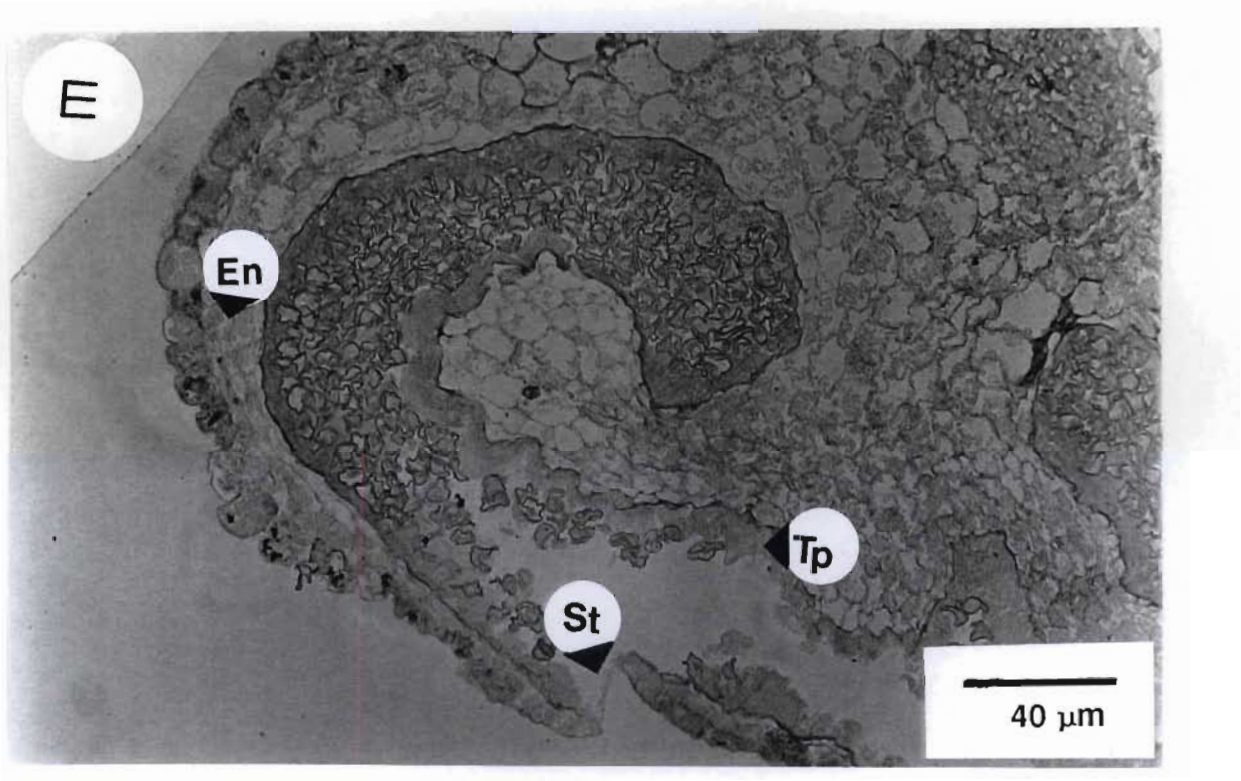


FIG 10.3


E
En
Tp
St
40 µm

3.3.4 Treatment 10:

The anthers of this treatment were transplanted to fresh medium after 28 days but were fixed after a further culture period of 28 days. The observations again agreed to a great extent with for of line 87.2002/3 (Treatment 10). In Figures 10.4 A - D it can be seen that the anther wall opened where the stomium had ruptured. The degeneration of the epidermis, endothecium, connective tissue and the tapetum had can be seen. Embryoid development and release from the pollen sporoderm occurred (Figures 10.4 C and D). A number of granular microspores were also observed, suggesting the absorbtion of starch and fatty acids from the tapetum.

Figure 10.4 Cross-section of Atzimba anthers which received a cold pre-treatment of 2 days, were cultured for 28 days on nutrient medium, then transferred to fresh medium and fixed after remaining for another 28 days on the fresh medium (Treatment 10) to indicate:

A - B. Connected pollen sacs open at the side of the anther lobe where the stomium broke. Largely degenerated epidermis, endothecium, tapetum and connective tissue.

C - D. Granular microspores, mature microspores enclosed within thick exine walls and sterile microspores are present. Embryoid release where exine ruptured. Limited embryoid development.

ct, connective tissue; **E**, epidermis; **Em**, embryoid; **ex**, exine; **GMs**, granular microspore; **in**, intine; **ml**, middle layer; **Ms**, microspore; **ps**, pollen sac; **pw**, pollen sporoderm; **SP**, sterile pollen; **St**, stomium; **Tp**, tapetum.

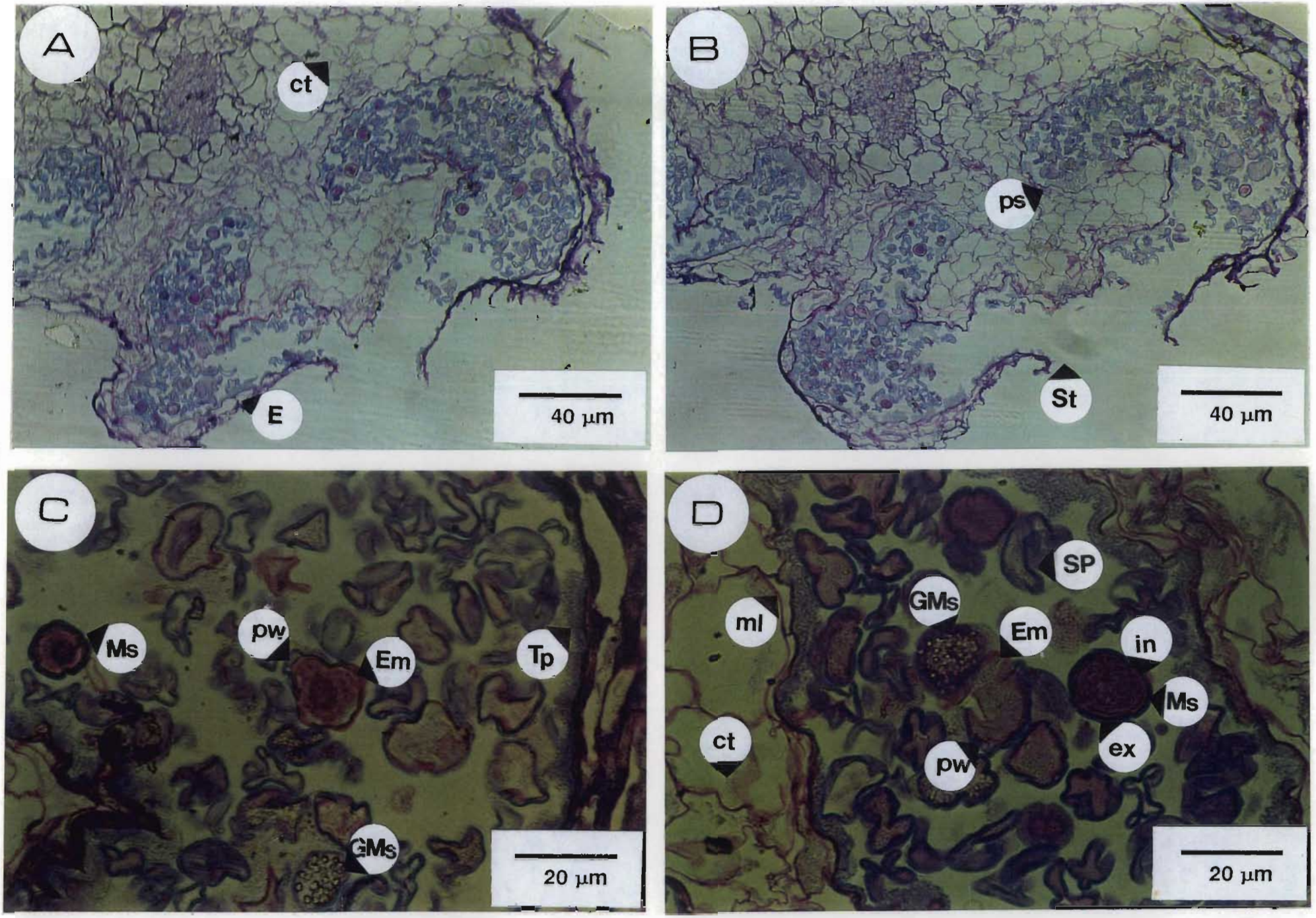

A
ct
E
40 µm
B
ps
St
40 µm
C
Ms
pw
Em
Tp
GMs
20 µm
D
SP
GMs
ml
Em
in
Ms
ct
ex
pw
20 µm

FIG 10.4

3.4 Interpretation of microtome sections of the anthers of diploid wild species *Solanum canasense*

3.4.1 Treatment 1:

Microspores were mainly in the uninucleate stage of development and were deformed in shape. The tapetum appeared relatively thin compared to its overall appearance in Atzimba and breeding line 87.2002/3.

3.4.2 Treatment 2:

As with the observations made for Atzimba anthers, the microspores of this treatment appeared more round in shape **after** the two day cold pre-treatment (compared to those before the cold treatment). This was in contrast with the observations made for line 87.2002/3. Most microspores were in the uninucleate stage of development, indicating that flower buds were selected at the correct stage for optimal androgenetic response.

Figure 11.1 Cross-section of *Solanum canasense* anthers fixed immediately after harvest, without a cold pre-treatment and without culturing on nutrient medium (Treatment 1) to indicate:

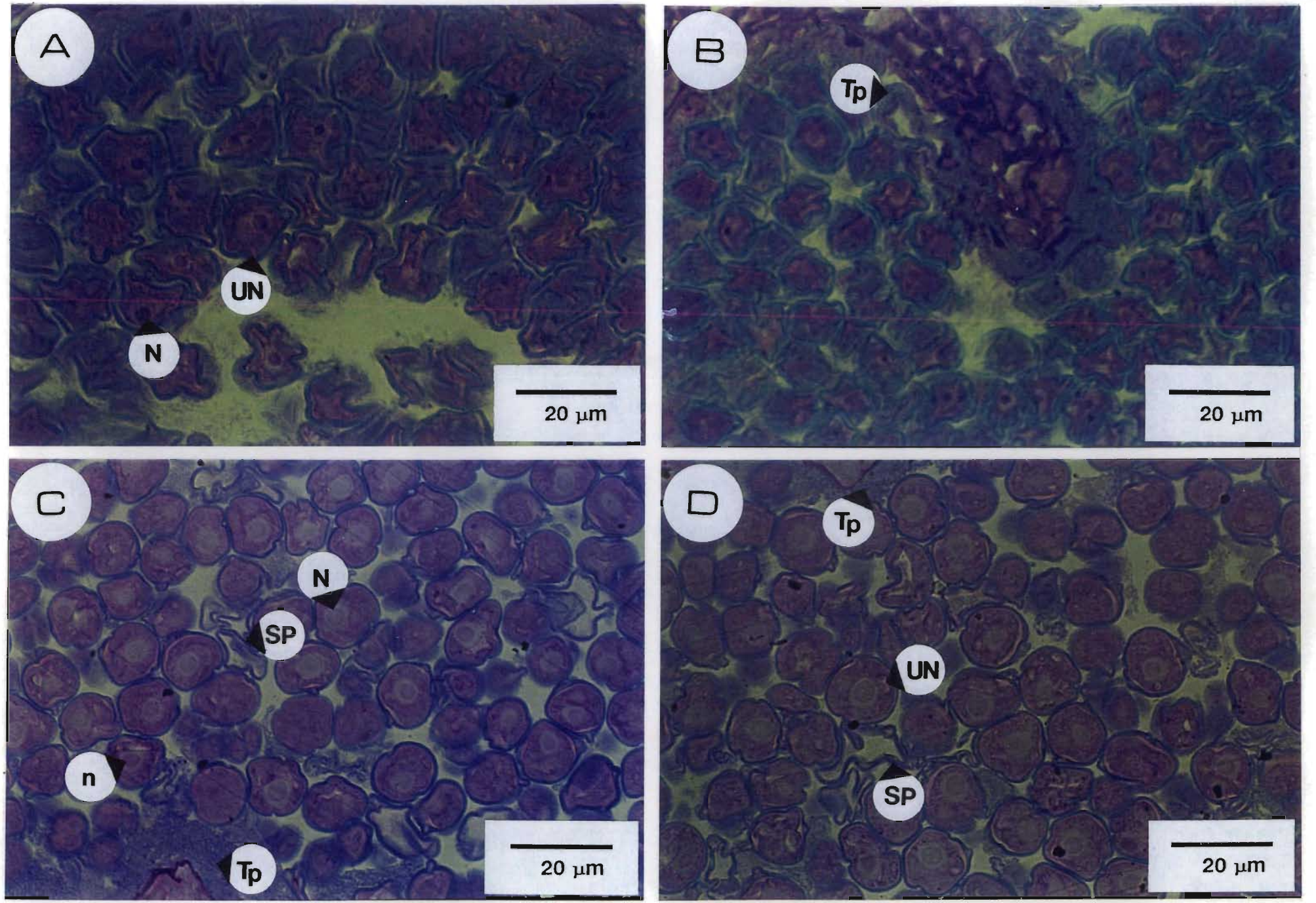
A - B. A relative thin tapetum. Approximately all microspores at the uninucleate stage of development. All microspores and other cells were deformed in shape.

N, nucleus; **Tp**, tapetum; **UN**, uninucleate microspores.

Figure 11.2 Cross-section of *Solanum canasense* anthers fixed after a cold pre-treatment of 2 days but <u>without</u> culturing on nutrient medium (Treatment 2) to indicate:

C - D. Microspores were all round in shape after the cold pre-treatment. Only uninucleate microspores could be distinguished and very little sterile pollen grains were present. The tapetum was well developed and relatively thick.

n, nucleolus; **N**, nucleus; **SP**, sterile pollen; **Tp**, tapetum; **UN**, uninucleate microspores.


A
UN
N
20 µm
B
Tp
20 µm
C
N
SP
n
Tp
20 µm
D
Tp
UN
SP
20 µm

3.4.3 Treatment 7:

The results from this treatment agreed to a great extent with that of Treatment 7 of both breeding line 87.2002/3 and Atzimba (as discussed). In Figure 11.3 A it can be seen that the pollen sacs contained a large number of sterile microspores. In some anthers, the anther wall appeared open where the stomium had ruptured, while in others the pollen sacs were not yet connected and the stomium appeared closed. Figure 11.3 A shows that even within the same anther, one side of the anther wall could already be open, while the other side remained closed. This could indicate the determining role of anther orientation on the earlier opening of the specific "side" of the anther which is in contact with the nutrient medium. This aspect will be addressed later. The cytoplasm was plasmolyzed and the epidermis, endothecium and connective tissue were largely degenerated. No embryogenic development was detected in any of the sections of this treatment.

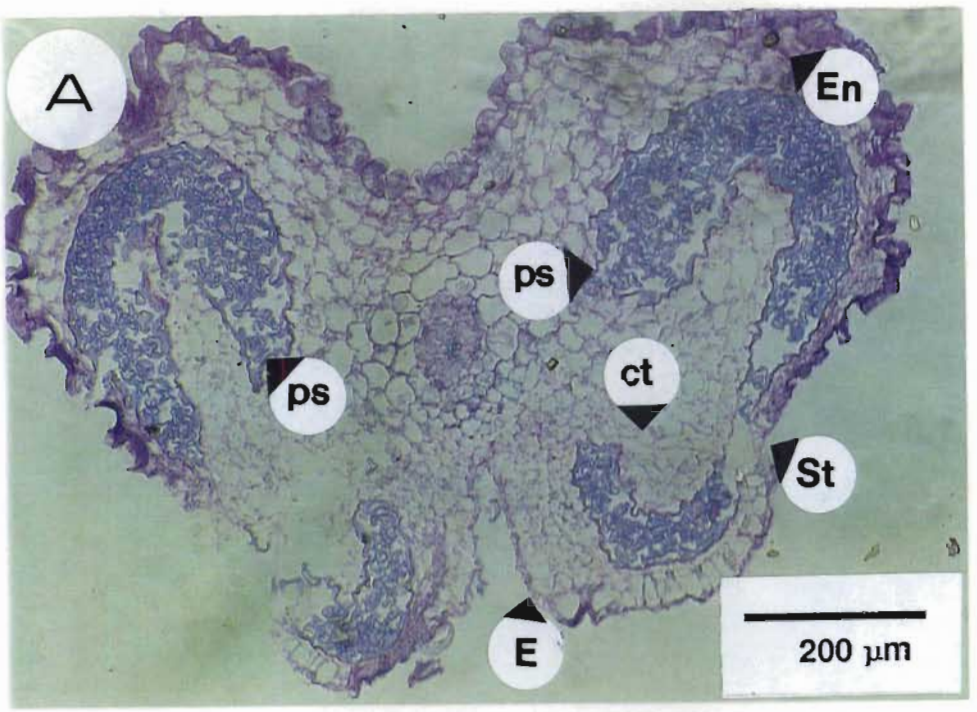
3.4.4 Treatment 10:

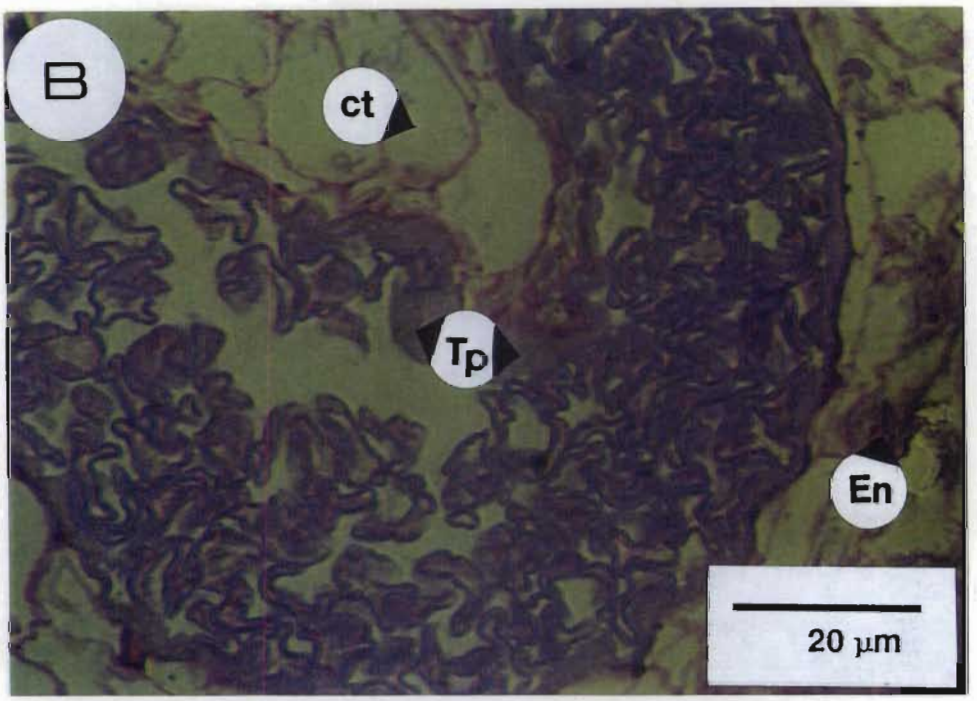
In Figures 11.4 A and B it can be seen that the process of degeneration of the cells was evident after a 56 day culture period. Furthermore, no embryogenic development occurred in this material, while embryoid release was detected in the anthers of both Atzimba and 87.2002/3 (Treatment 10). These observations could imply that *Solanum canasense* is androgenetically unresponsive. The latter could also be connected to the fact that the tapetum appeared relatively thin compared to that of Atzimba and 87.2002/3, thus suggesting that the tapetum could not effectively supply nutrients to the developing microspores. However, a limited number of granular microspores occurred (Figures 11.4 C and D), suggesting that starch and fatty acids were absorbed from the tapetum. The

Figure 11.3 Cross-section of *Solanum canasense* anthers fixed after a cold pre-treatment of 2 days and a culture period of 35 days on nutrient medium (Treatment 7) to indicate:

A. A cross-section of an anther showing the two pollen sacs fused in each of the two anther lobes, a large number of sterile pollen and degenerating connective tissue and epidermis. The stomium is quite prominent at the right half where the two pollen sacs have not yet fused, while the left anther lobe already opened for microspore release.
B - C. Thin, degenerating tapetum, a few granular microspores and many sterile microspores in the pollen sacs. Elongated cells of endothecium and rounder cells of connective tissue surrounding the pollen sac.

ct, connective tissue; **E**, epidermis; **En**, endothecium; **GMs**, granular microspore; **ps**, pollen sac; **SP**, sterile pollen; **St**, stomium; **Tp**, tapetum.


A
En
ps
ps
ct
St
E
200 µm


B
ct
Tp
En
20 µm

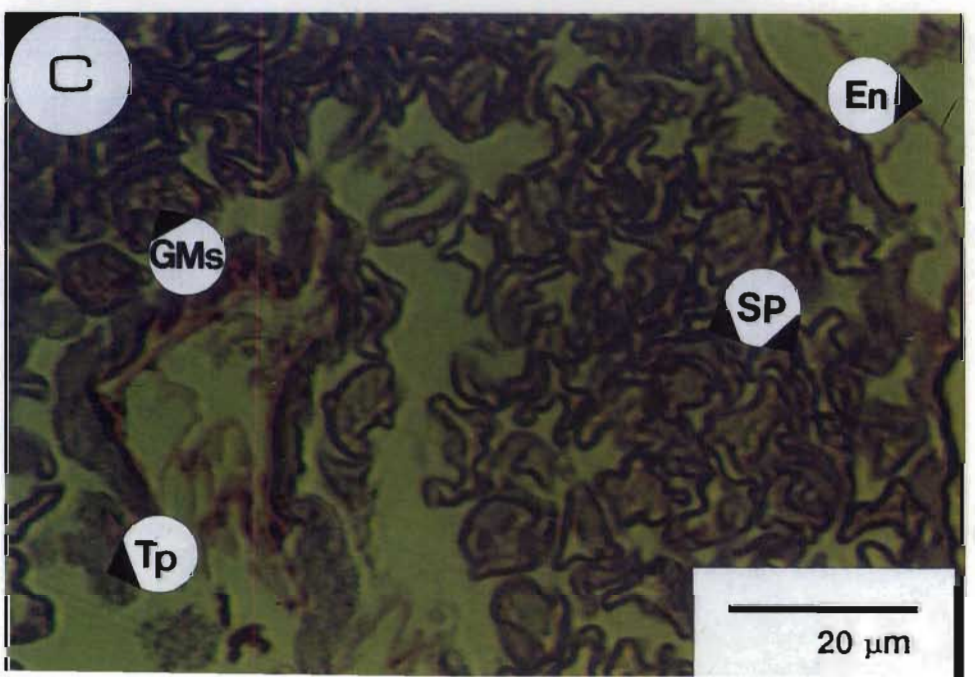
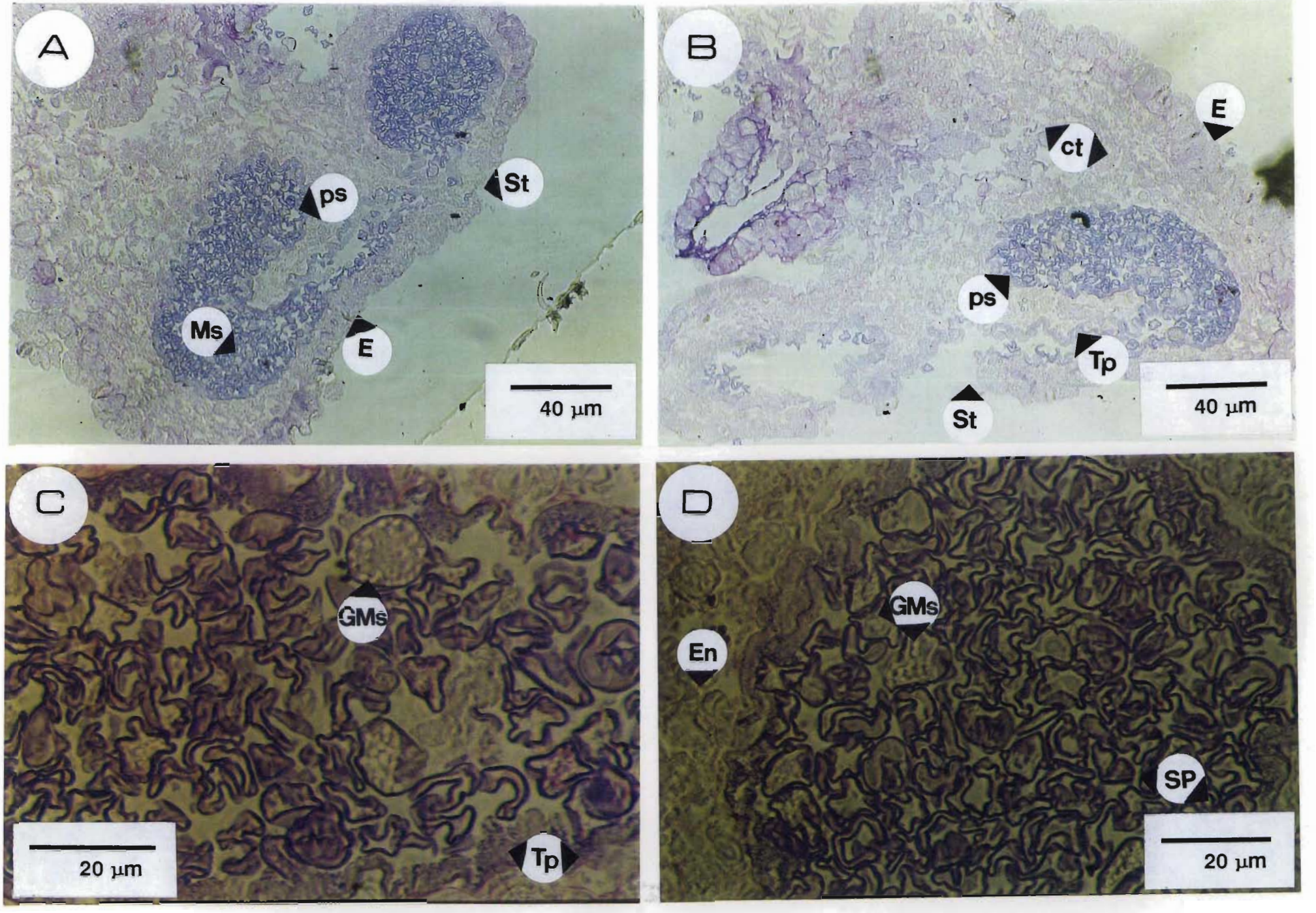

C
En
GMs
SP
Tp
20 µm

Figure 11.4 Cross-section of *Solanum canasense* anthers which received cold pre-treatment of 2 days, were cultured for 28 days on nutrient medium, then transplanted to fresh medium and fixed after remaining for another 28 days on the fresh medium (Treatment 10) to indicate:

A - B. Connected pollen sacs open at the side of the anther lobe where stomium broke. Extremely degenerated epidermis, endothecium, connective tissue and tapetum.

C - D. Mainly granular and sterile microspores, surrounded by thin degenerating tapetum. No embryoid development was found.

ct, connective tissue; **E**, epidermis; **En**, endothecium; **GMs**, granular microspore; **Ms**, microspores; **ps**, pollen sac; **SP**, sterile pollen; **St**, stomium; **Tp**, tapetum.


A
ps
St
Ms
E
40 µm
B
E
ct
ps
Tp
St
40 µm
C
GMs
20 µm
Tp
D
GMs
En
SP
20 µm

latter could imply, therefore, that - although not effective enough - the tapetum supplied nutrients to the microspores. The microspores showed no signs of embryogenic development.

IV CONCLUSIONS

This microscopic study of fixed material confirmed that the anthers from all three genotypes possessed the normal anatomy and development typical of the family Solanaceae (FAHN, 1974). The development of the endothecium bands (as indicated in sections of line 87.2002/3) and subsequent opening of the anther wall at the stomium (87.2002/3, Atzimba and *Solanum canasense*) also agreed with the descriptions by FAHN (1974). The active nutritive function of the tapetum is implied by the sections made. The rapid degeneration of the tapetum as microspore and eventually embryoid development progressed, supports this nutritive role. The relative thickness of the tapetum varied between the genotypes studied and even between different anthers of the same genotype. This variation could be of genetic origin and could possibly explain why some anthers/genotypes were androgenetically more responsive. It can be argued, for instance, that the developing microspores would probably receive nutrients more effectively where the tapetum appeared well developed and thick, compared to those surrounded by an underdeveloped, thin tapetum. Thus, the thickness and efficiency of the tapetum could induce differences in microspore development in cultured anthers. According to DUNWELL (1985) the microspores closest to the tapetum will receive more nutrients than those situated in the centre and furthest from the tapetum. This in some cases could, even cause a nutrient deficiency in the developing microspores.

The overall effectiveness of the tapetum of breeding line 87.2002/3 and

the efficient absorbtion of nutrients from the medium could be concluded from the fact that embryogenesis was induced within the first seven days of the culture period of this breeding line. The opposite proved true, however, for the diploid wild species *S. canasense* where the tapetum appeared relatively thin and no embryoid development was observed.

In some instances the stomium appeared closed in an anther, while in others (òr the same) of the same treatment, the anther wall had already ruptured at the stomium opening. This difference within the same treatment (and/or within the same anther) could either be due to the fact that dehydration was not synchronized in all the material (probably due to unsynchronized anther development), or due to the possibility that more stomata occurred at the lateral "sides" of the anther. If the latter was true, it can be argued that where the stomium appeared open, it could be that that specific lateral side of the anther was in contact with the nutrient medium, leading to the earlier rupture of the anther wall and consequent release of developing microspores/embryoids onto the medium. The question of anther orientation was investigated and will be addressed in Chapter 3.

The optimal microspore developmental stage for androgenetic response, namely the uninucleate stage, was observed, as were the binucleate and late binucleate stages and sterile microspores. The occurrence of both uni- and binucleate microspores within the same anther/pollen sac, confirmed that the active process of mitosis was occurring in the cells and thus the fertility of the microspores in the cultured anthers. It can also be concluded that the flowers of the dihaploid line 87.2002/3 and wild species *S. canasense* were selected at approximately the correct microspore developmental stage. The flowers of 87.2002/3 could even have been selected a fraction earlier, due to the large number of microspores in the binucleate developmental stage. However, no starch depositioning was observed in these microspores, indicating that the

time of flower bud selection was not too late. On the other hand the Atzimba anthers could have been selected too late due to the occurrence of starch depositioning even before the culture period commenced. According to HEBERLE-BORS (1982) starch depositioning occur during the **late binucleate stage** of microspore development.

PIERIK's assumption (1993), that the development of the microspores contained in any one anther is not synchronized, was confirmed by the results from the microscopic study for all three genotypes. Both uninucleate and binucleate microspores occurred within the same anther (as found in 87.2002/3, Atzimba and *S. canasense*); òr both mature, uninucleate microspores, multicellular microspores (young, developing embryoids) and embryoids at the globular development stage occurred within the same anther and even the same pollen sac (as found in 87.2002/3). This aspect was also confirmed by the results from Experiment 2 where both microspore mother cells, diades and tetrads; or tetrads, uninucleate and binucleate microspores occurred within the same anther. It is recommended to first determine when most microspores in an anther are in the uninucleate stage of microspore development to obtain optimal androgenetic response from responsive potato genotypes. The DAPI-technique proved an efficient and rapid method for the determination of microspore developmental stages.

The general variation that occurred during anther culture of different potato genotypes, can be due to various factors. For instance, potato flower buds were not all selected from the same donor plants during each of the various replications of the experiments òr glass house and/or medium conditions could have varied during the different trials. Optimal androgenetic response was found in those anthers selected not later than the first 7 to 21 days of the flowering period. Variation in response can, therefore, be induced when flowers are selected for the duration of the flowering period from the same donor plant for

experiments consisting of several flower selections. This continous selection could lead to the inclusion of both "younger/first" flowers, as well as "older" flowers (selected later in the flowering period).

The fact that not all the microspores present in an anther developed during the culture period could either be a genetic effect or alternatively the medium conditions utilized were not optimal. The occurrence of sterile microspores as well as microbial contamination within anthers, would lead to a general reduction in the androgenetic response.

Finally, even if most microspores, present in the anthers of selected flower buds, are in the uninucleate (or the specific required optimal) microspore developmental stage and the flowers were all selected during the first 7 - 21 days of the flowering period, embryogenesis would only occur within those genotypes which are genetically **amenable to in vitro anther culture**. This only provided that optimal culture conditions are utilized to satisfy the specific requirements of the material used. The aspect of optimization of the nutrient medium and culture conditions (e.g. the culture phase, hormone concentrations and anther orientations) were investigated and will be reported on in following Chapters.

CHAPTER 3

ALTERNATIVE CULTURE METHODS

I INTRODUCTION

CHASE (1963) proposed an analytical breeding scheme for potatoes in which dihaploids of *Solanum tuberosum* ssp. *tuberosum* (2n=4x=48) are utilized for breeding with wild or cultivated diploid species (SNIDER and VEILLEUX, 1994). Hereby, heterozygosity can be increased in the cultivated forms. Previously, diploid *S. tuberosum* (2n=2x=24) was obtained primarily via parthenogenesis, while anther culture techniques (androgenesis) proved more successful for obtaining true haploids from diploid species (FOROUGHI-WEHR, WILSON, MIX and GAUL, 1977; SOPORY, JACOBSEN and WENZEL, 1978; WENZEL and UHRIG, 1981). Anther cultures are, however, now more commonly used and the production of haploids has been reported for different members of the family *Solanaceae* (JOHANSSON, 1986). *S. tuberosum* is an important exception and according to DUNWELL and SUNDERLAND (1973), JOHANSSON (1983) and MIX (1983) there are only a few reports of successful induction of androgenesis that led to fully developed dihaploid individuals from tetraploid potato genotypes. JOHANSSON (1986) and SNIDER and VEILLEUX (1994), however, reported embryogenesis and the production of dihaploid plants from anther cultures of several tetraploid *S. tuberosum* clones. According to JOHANSSON (1986) this success, as well as an overall increase in embryogenesis in anther cultures of *Solanum tuberosum*, can be ascribed to the correct treatment of the donor plants, pretreatment of flower buds and manipulation of the culture media. The determining role of these and other variables on androgenetic response which all serve as sources of variation for embryoid production, was emphasized by several authors (VASIL, AHUJA

and VASIL, 1979; MAHESHWARI, TYAGI and MALHORTA, 1980; MAHESHWARI, RASHID and TYAGI, 1982; WENZEL and FOROUGHI-WEHR, 1984; DUNWELL, 1985; SOPORY and BAJAJ, 1987). For successful dihaploid production by means of androgenesis, these sources of variation need to be identified and controlled (SNIDER and VEILLEUX, 1994). This study focussed on the establishment of optimal culture conditions and requirements by analyzing and comparing alternative culture methods which could lead to an increase in androgenetic response in different potato genotypes. Both the effect of different culture phases and different anther orientations were investigated.

Liquid (UHRIG, 1985) and double layered culture media with activated charcoal (JOHANSSON, 1986) enhanced the ability of anthers to dehisce spontaneously and shed pollen and pollen fractions into the surrounding fluid to form embryoids ("shed-pollen" technique). If the liquid portion of double layered cultures was thin enough, pollen would either remain in the upper layer and develop into embryoids, or would settle on the lower agar-solidified layer and develop there (JOHANSSON, 1986). Both liquid and double layered cultures have the advantage that all media components are easily accessible to developing embryoids, thus reducing competition (UHRIG, 1985; JCHANSSON, 1986). Furthermore, with double layered cultures, inhibitors produced by degenerating anthers can diffuse and be absorbed by the underlying solidified charcoal layer (JOHANSSON, 1986). This so-called "shed-pollen" technique produced much larger numbers of embryoids compared to the ordinary culture of anthers in solid medium (MAHESHWARI, RASHID and TYAGI, 1982).

With the utilization of liquid culture media, the effect of a specific orientation of cultured anthers is undefined, as anthers tend to float on the surface of the medium (DUNWELL, 1985). With agar-solidified media, however, the orientation of the anthers can be controlled and the

question, as to whether different anther orientations could significantly affect the yield of microspore derived embryoids, arises. Anther orientation proved a determining variable in several crops, such as *Datura* and *Nicotiana* (SOPORY and MAHESHWARI, 1976) and *Hordeum* (HUNTER, 1985; SHANNON, NICHOLSON, DUNWELL and DAVIES, 1985; POWELL, BORRINO and GOODALL, 1988). The best orientation varies with different species (HUNTER, 1985). In *Hordeum vulgare* L. lateral orientation (with one lobe in contact with the culture medium) significantly increased the number of plantlets produced (HUNTER, 1985; SHANNON, NICHOLSON, DUNWELL and DAVIES, 1985). Microspores contained in the upper half of these lateral orientated anthers, normally developed to produce embryoids or callus-like structures, while those in the lower half normally ceased growth. This difference in response between the two lobes, underlined the importance of the addition of specific required growth regulators, especially during the later culture period, as the medium is transferred to these cells by means of diffusion (DUNWELL, 1985). This conclusion was reached after DUNWELL (1985) reported the inhibitory effect of 2,4-D and that the difference in response between the lobes decreased after the kinetin and 2,4-D present in the initial basal medium, were replaced with indole-acetic acid and benzyladenine. According to DUNWELL (1985) and HUNTER (1985), the "flat" orientation, in general, resulted in shedding of the microspores onto the medium whereafter no further development occurred. Furthermore, embryoids developed mostly from anthers in the lateral orientation, while with the flat orientation callus was usually produced (DUNWELL, 1985). Embryoid production is preferred as the ploidy level of these plantlets is normally stable, while the ploidy level of plantlets regenerated from callus tissue normally varies and somaclonal variation can occur. The above authors only distinguished between the lateral orientation (with one lobe in contact with the culture medium) and the "flat" orientation (where both lobes were in contact with the medium). No distinction was made, however, as to whether the two lobes involving the upper part of the

anther or those involving the lower part of the anther were in contact with the medium.

Two experiments were conducted in this study to investigate the effect of the two mentioned variables. In the first experiment the effect of three different culture phases (liquid, double layered and standard agar-solidified) on the androgenetic response was investigated. The nutrient composition remained basically the same. The second experiment investigated the effect of three different anther orientations on the yield of microspore derived embryoids. The genotypes incorporated into this study included a local tetraploid cultivar (Atzimba) with limited androgenetic response, dihaploid breeding lines (87.2002/3 and 87.2002/6) selected for their high androgenetic response and a diploid wild species (*S. canasense*) with no reported androgenetic tendency.

II MATERIALS AND METHODS

1. Plant Material

Virus free potato tubers of selected genotypes were grown in a glass house under controlled conditions. A light intensity of 200 µmol $m^{-2}s^{-1}$, day/night temperature of 21°C/14°C and a 16 h photoperiod proved to be essential for optimum flower formation. This light instensity could vary greatly in the glass houses used. Material from the commercial tetraploid cultivar Atzimba, the diploid wild species *S. canasense* and dihaploid breeding line 87.2002/3 were selected for both experiments. For the second experiment, anthers from another dihaploid breeding line, 87.2002/6, were also cultured. These two dihaploid breeding lines, obtained from the Max Planck Institute for Plant Breeding, Köln, have superior androgenetic ability and large numbers of embryoids are produced directly by the pollen of these lines.

2. Anther Culture

2.1 Culture Procedure and Media Composition

For maximum response in anther culture, anthers have to be in the uninucleate stage of microspore development, that is free of starch (WENZEL and FOROUGHI-WEHR, 1984). Flower buds with a length of 4.2 - 4.5 mm were selected and received a cold pretreatment of 6 - 9°C for at least 48h prior to culture (WENZEL and FOROUGHI-WEHR, 1984). The optimal flower bud lengths vary between different potato genotypes and can be pre-determined by means of fluorescent techniques as described in Chapter 2. The flower buds were then surface sterilized in 70% ethanol for two to three minutes, followed by 10 minutes in 1% sodium hypochlorite with 0.1% Tween-20 added and subsequently washed three times in sterile, distilled water. The anthers were dissected under aseptic conditions from the flower buds and plated on standard agar-solidified initiation medium in 90 x 10 mm sterile, plastic petri-dishes (Figure 12 A - B). Before incubation the petri-dishes were sealed with a double layer of parafilm.

The standard initiation medium (Table 12[a]) consisted of the basal medium of LINSMAIER and SKOOG (1965) at half strength, supplemented with sucrose, agar and the indicated filter-sterilized growth regulators. Developed embryoids were transferred to an embryoid-regeneration medium (Table 12[b]) (Figure 12 C). As soon as the embryoids formed roots or started to turn green, they were transferred to an embryoid maturation medium (Table 12[c]). After sufficient development, plantlets were transferred to test tubes containing a modified MURASHIGE and SKOOG medium (1962) (referred to as Osborne medium) (Figure 12 D). After reaching a height of about 8 cm, rooted plantlets were transferred to sterile soil in pots under glasshouse conditions (Figure 12 E). The ploidy of developed plantlets was determined by counting the number of chloroplasts in stomatal guard cells (FRANDSEN, 1968 as quoted by

MEYER, SALAMINI and UHRIG, 1993) (Figure 13). As the number of chloroplasts differ in the various stomatal guard cells, an average should be determined. The ploidy level can be confirmed by means of chromosome countings from squashed root tips.

In the event of callus formation from microspores, the calli were transferred to a shoot regeneration medium (Table 12[d]). Regenerated plantlets were transferred to Osborne medium (Table 12) in test tubes (as previously discussed) and eventually rooted plantlets were established in soil.

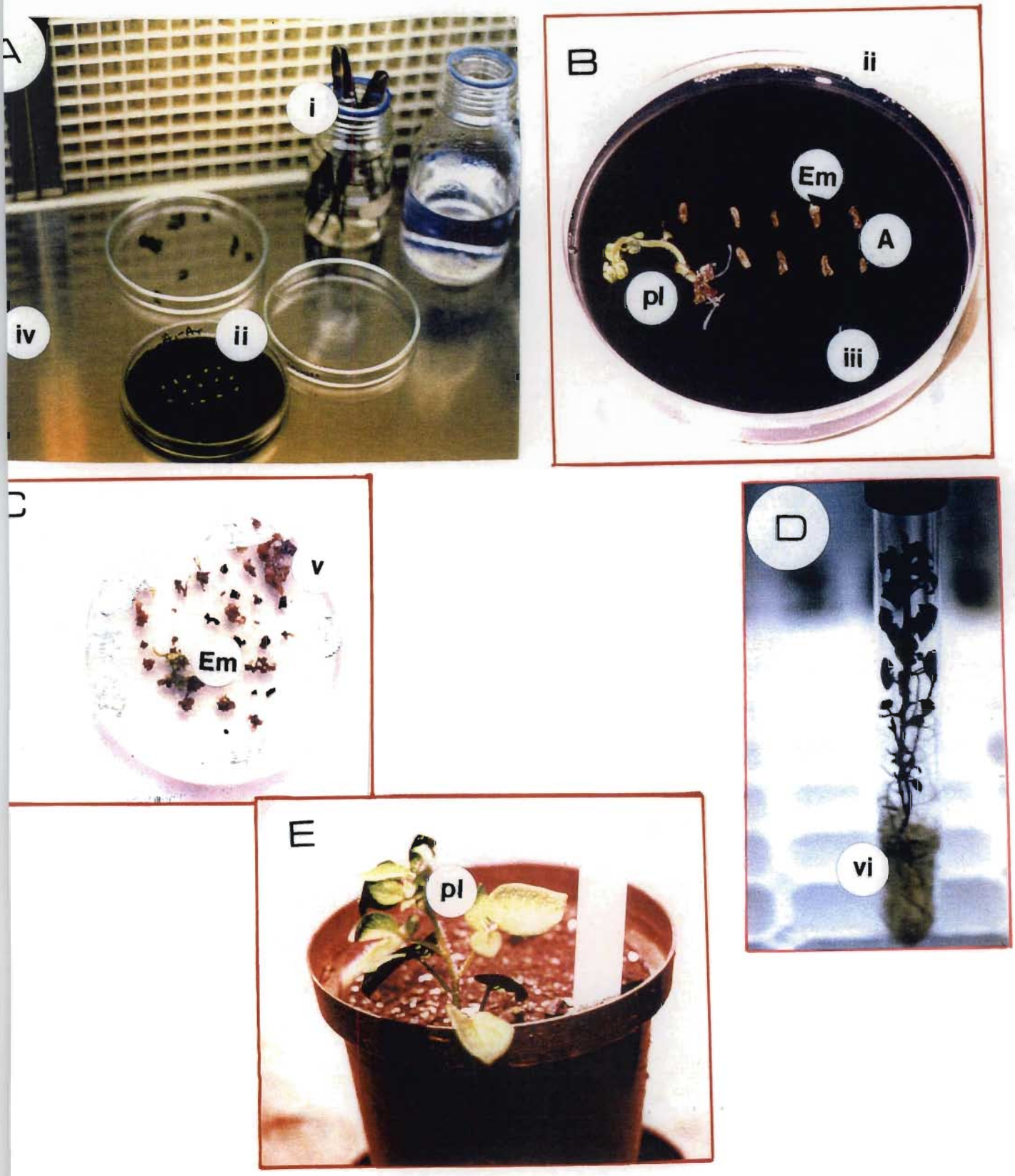
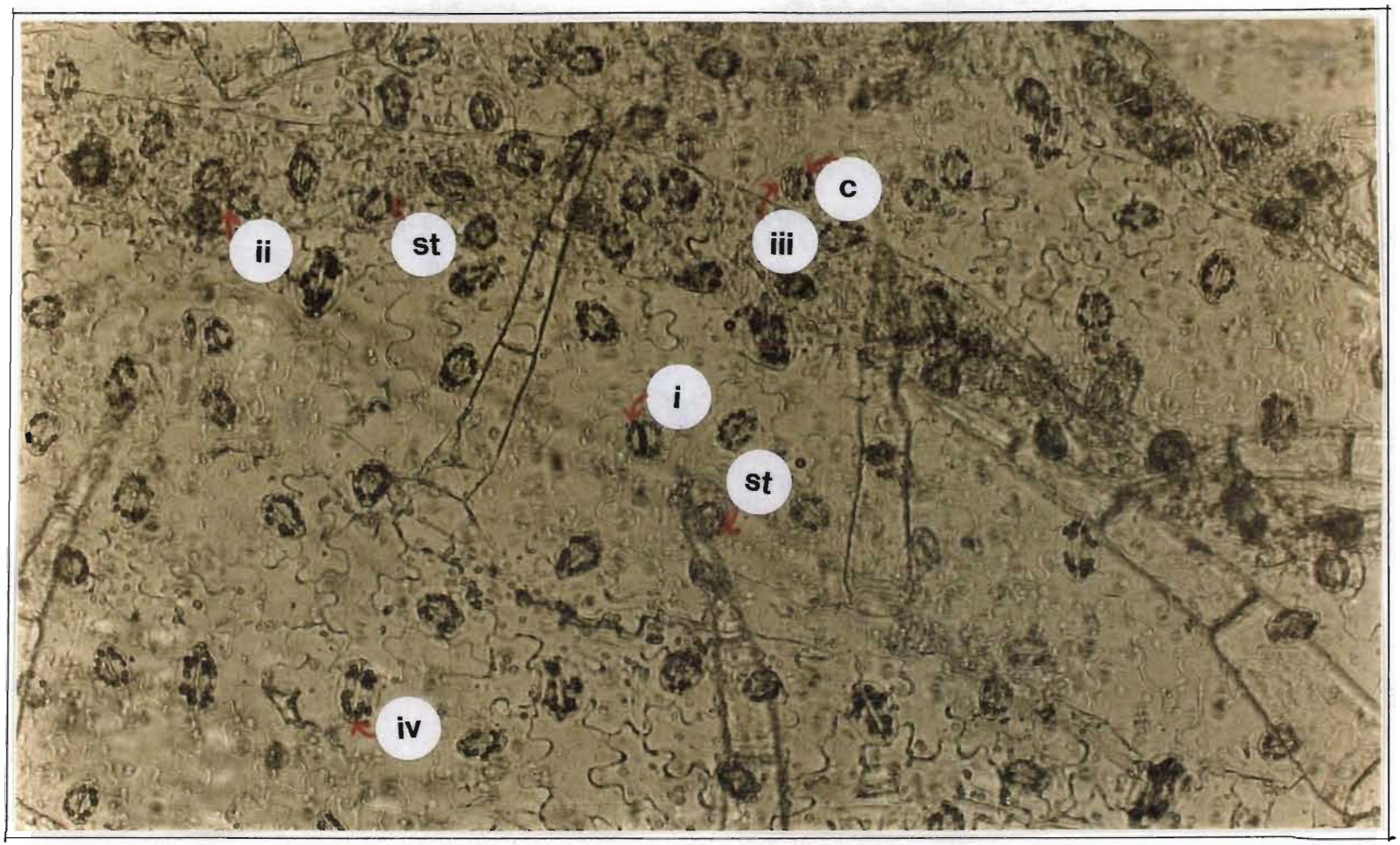


Figure 12 Summary of basic anther culture techniques, indicating:

A. The culture of anthers from three potato flower buds on standard initiation medium Table 1[a], containing activated charcoal, under aseptic conditions in a laminar flow cabinet.
B. Anthers from two flower buds, cultured on initiation medium [a]. White, globular microspore derived embryoids are visible on the surface of some anthers as well as a direct microspore derived plantlet.
C. Embryoids transferred to an embryoid regeneration medium for root- and shoot formation. Some embryoids died (brown) while a few developed into green plantlets.
D. Developed plantlet transferred to Osborne medium in a test tube for multiplication, long term *in vitro* maintenance and, eventually;
E. the transfer of rooted plantlets to sterile soil in pots under glass house conditions for tuber formation and ploidy determination.
i, sterile forceps in alcohol; **ii**, petri-dish containing dissected anthers, plated on **iii**, initiation medium; **iv**, laminar flow cabinet surface; **v**, embryoid regeneration medium; **vi**, test tube containing Osborne medium; **A**, anthers; **Em**, embryoids; **pl**, plantlet.



gure 13 Micrograph showing the sub-epidermal layer of a leaf from a microspore derived plantlet of breeding line 87.2002/3, confirming the dihaploid nature of the plantlet:

(i) stomatal guard cell containing 12 chloroplasts;
(ii) stomatal guard cell containing 10 chloroplasts;
(iii) stomatal guard cell containing 10 chloroplasts;
(iv) stomatal guard cell containing 13 chloroplasts;

c, chloroplast; **st**, stomium

Table 12 Composition of initiation, regeneration and maturation media utilized in potato anther culture experiments

Composition	Media used				
	[a]	[b]	[c]	[d]	Osborne
LS	half strength	half strength	half strength	full strength	full strength
Thiamine HCl	0.2 mg l^{-1}	0.2 mg l^{-1}	0.2 mg l^{-1}	-	-
(myo)-Inositol	50 mg l^{-1}	50 mg l^{-1}	50 mg l^{-1}	-	-
Benzyladenine (BA)	1.0 mg l^{-1}	-	-	-	-
Indole-3-acetic acid (IAA)	0.1 mg l^{-1}	-	-	-	-
Asparagine	7.5 mg l^{-1}	-	-	-	-
Glutamine	7.0 mg l^{-1}	-	-	-	-
Gibberellic acid (GA_3)	-	0.1 mg l^{-1}	-	0.02 mg l^{-1}	0.1 mg l^{-1}
Naphtalene-1-acetic acid (NAA)	-	-	-	0.02 mg l^{-1}	0.01 mg1^{-1}
Zeatin	-	-	-	2.0 mg l^{-1}	-
Glycine	-	-	-	2.0 mg l^{-1}	-
Activated charcoal	5 g l^{-1}	-	-	-	-
Agar	8 g l^{-1}	8 gl^{-1}	8 g l^{-1}	8 g l^{-1}	7 g l^{-1}
Sucrose	60 g l^{-1}	20 gl^{-1}	20 g l^{-1}	20 g l^{-1}	30 g l^{-1}
pH	5.8	5.8	5.8	5.8	5.5

LS = basal medium of LINSMAIER and SKOOG (1965)
[a] = initiation medium;
[b] = embryoid regeneration medium;
[c] = embryoid maturation medium
[d] = shoot regeneration medium

Experiment 1: Liquid- vs double layered vs standard agar solidified media.

Anthers of Atzimba, *S. canasense* and 87.2002/3 were each initially cultured for one week on initiation medium (Table 12[a]) to determine if the anthers were contaminated internally. The five anthers from one

flower bud were cultured together in a petri-dish. After one week anthers were transferred in equal numbers to the three different types of media.

Standard Cultures

These cultures served as the control treatments. The five anthers contained in one flower bud were plated in a petri-dish on approximately 20 ml initiation medium (Table 12[a]). Anthers were all orientated laterally, that is with one lobe in contact with the culture medium. Cultures were incubated at 24°C using a 12 h photoperiod, 60-80% humudity and a light intensity of 400 µmol $m^{-2}s^{-1}$.

Liquid Cultures

The liquid medium consisted of the basal medium of LINSMAIER and SKOOG (1965) at half strength, supplemented with the same organic substances and filter-sterilized growth regulators indicated in the initiation medium (Table 12[a]) but with a lower activated charcoal concentration of 0.5 g l^{-1}. Approximately 10 ml of liquid medium were added per sterile 50 ml Erlenmeyer flask. The five anthers plated per petri-dish (all five originating from the same flower bud) were transferred to a flask and the flasks were sealed with a double layer of aluminium foil and parafilm. The cultures were incubated on a shaker, rotating at 100 r.p.m., in a growth cabinet at 25°C in the dark. Developed embryoids were harvested by transferring the contents of the Erlenmeyer flask to a sterile petri-dish and removing the embryoids aseptically from the petri-dish. The harvested embryoids were cultured on regeneration medium Table 12[b]. Anthers were again incubated in a sterile flask together with the remaining portion of the liquid medium and, depending on the specific condition of each culture, 5 - 10 ml of fresh liquid medium were added. The original liquid portion had to be re-utilized as microscopic small embryoids could already be present in this medium and would be lost if discarded. If no embryoids were produced after four weeks, 5 ml of fresh liquid medium were added to each culture.

Double Layered Cultures

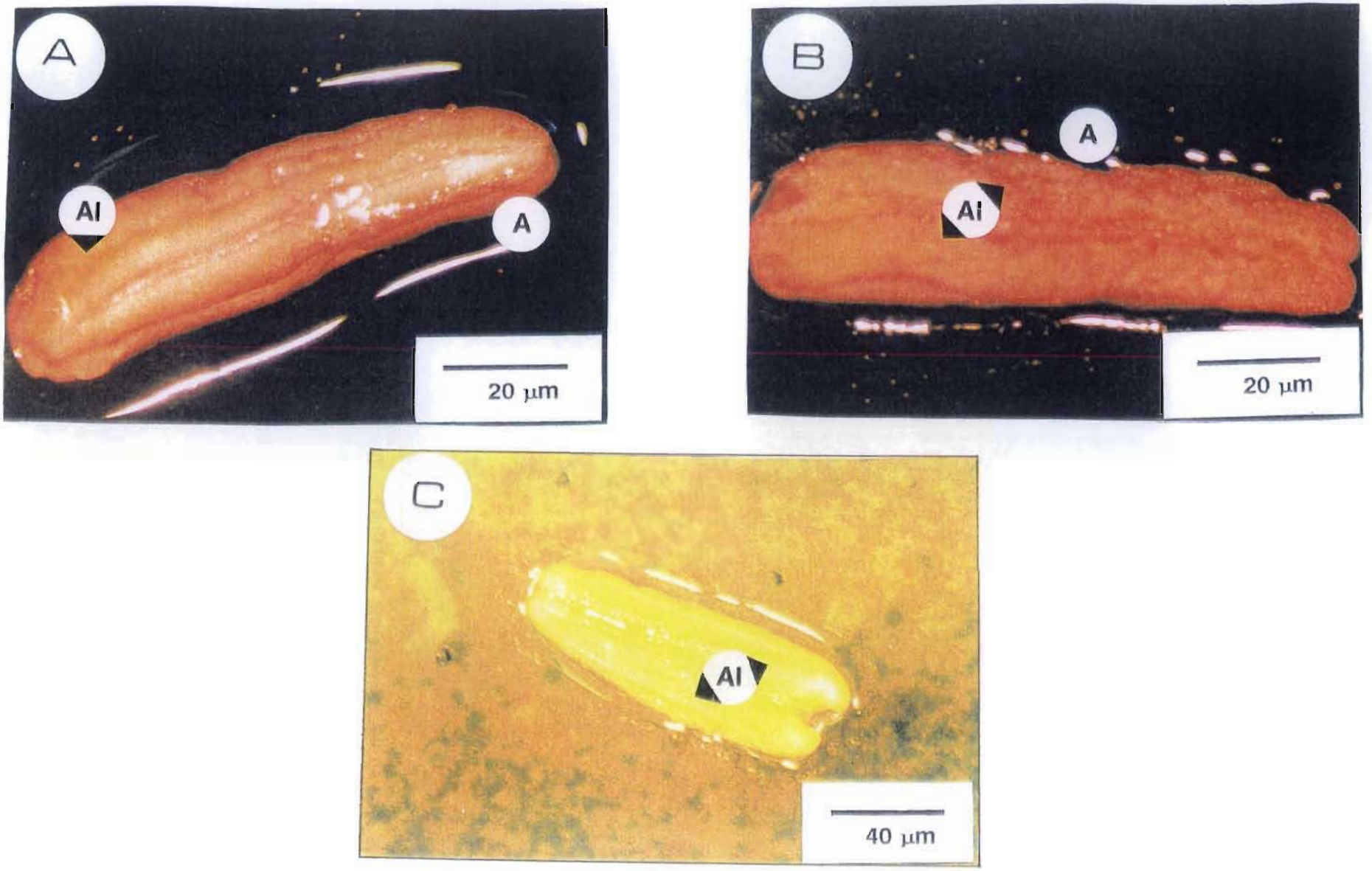
These cultures consisted of a base layer of initiation medium Table 12[a] but without additional filter-sterilized growth regulators, and a top layer of 5 ml liquid medium. The liquid medium used was the same as the liquid medium outlined above, but it contained no activated charcoal. Filter-sterilized growth regulators were added just before use. Five anthers were cultured per petri-dish. Developed embryoids were harvested from responding anthers and transferred to regeneration medium Table 12[b], after which the anthers were placed on fresh double layered medium. If no embryoids were produced, 2-3 ml of fresh liquid medium was added to the cultures.

Experiment 2: Anther orientation

Anthers of selected genotypes Atzimba, *S. canasense*, 87.2002/3 and 87.2002/6 were cultured, respectively, dorsally (Figure 14 A) (with the dorsal side of the two lobes in contact with the medium), ventrally (Figure 14 B) (with the ventral side of the two lobes in contact with the medium) and laterally (with only one lobe in contact with the medium) (Figure 14 C). The five anthers from each flower bud were cultured together in a petri-dish on initiation medium (Table 12[a]). An equal number of anthers of each genotype were cultured per orientation.

2.2 Statistical Analysis

Complete randomised designs were used in all experiments. Results were recorded every four weeks. The number of responding anthers as well as the total number of embryoids produced were taken into account. From this data the variables, embryoid frequency (amount of embryoids produced in 100 anthers), anther response (% anthers that form embryoids) and anther productivity (number of embryoids produced from responding anthers), were determined. For the first experiment, data was analyzed with the general linear model (GLM), using the Poisson distribution. Data from the second experiment was log-



gure 14 (A-C). Anthers from dihaploid breeding line 87.2002/3 cultured on initiation medium to indicate the three different anther orientations:

A. Anther orientated laterally with only one anther lobe in contact with the culture medium.
B. The dorsal orientation with the dorsal side of the two anther lobes in contact with the culture medium.
C. An anther orientated ventrally with the ventral side of the two anther lobes in contact with the culture medium.

A, anther; **Al**, anther lobe(s).

transformed and analyzed with the general linear model (GLM), using the normal distribution. For all three variables investigated (embryoid frequency, anther response and anther productivity) all differences reported were significant ($P < 0.05$), unless otherwise indicated.

III RESULTS

Experiment 1: Liquid- vs double layered vs standard agar solidified media.

This experiment was conducted twice. The first trial commenced in 1993 and the second in 1994. From the material incorporated into this study, the diploid wild species *S. canasense* showed no response and data was analyzed only for the tetraploid cultivar Atzimba and dihaploid breeding line 87.2002/3. The differences in response between the two entries (Atzimba and 87.2002/3), the three culture medium phases (liquid, double layer and standard agar solidified media) and the interaction between the two main effects "entries" and "medium" were analyzed.

The interaction between the treatments entries and media was significant in terms of embryoid frequency (Table 13d). No significant differences in terms of embryoid frequency were found between the two entries (Table 13b) Atzimba (1.28) and 87.2002/3 (2.10). Although the differences in the main effect medium (Table 13c) were significant, only the interaction is of value. The interaction (Table 13d) showed that for cultivar Atzimba the liquid medium (3.85) yielded significantly higher embryoid frequencies than both double layer (0.00017) and standard medium (1.16), while the standard medium gave significantly higher yields compared to the double layer medium. Breeding line 87.2002/3 showed a higher embryoid frequency with the utilization of standard medium (5.06), compared to both the double layer (0.56) and liquid

media (0.00017). No significant difference was observed between the liquid and double layer medium for line 87.2002/3.

In terms of anther response the interaction between the two treatments entries and medium was significant (Table 14d). Differences were also significant between both main effects entries (Table 14b) and medium (Table 14c), but only the interaction is of value. The interaction (Table 14d) indicated that for cultivar Atzimba, both the liquid (0.44) and standard media (0.41) initiated a significantly higher anther response compared to the double layer medium (0.0005). No significant differences were found, however, between the liquid and standard medium. For line 87.2002/3 the highest anther response was observed when the standard medium (1.93) was used compared to both double layer (0.29) and liquid media (0.0005).

It can be seen that for cultivar Atzimba, more embryoids were formed (3.85, Table 13d) - from approximately the same amount of anthers - under liquid culture conditions (0.44, Table 14d) compared to that for standard medium conditions where only 1.16 (Table 13d) embryoids were produced (0.41, Table 14d). This is confirmed by the production index (embryoid frequency LSMEAN / anther response LSMEAN) where the liquid medium had the highest index (8.75), followed by the standard medium (2.83) and double layer medium with the lowest index (0.34). For line 87.2002/3 the standard agar solidified medium gave the highest index value (2.62) followed by the double layer medium (1.93) and liquid medium with the lowest index (0.34).

In terms of anther productivity the differences between the three different media treatments were not significant (Table 15b). This implies that, once anthers have been conditioned for embryoid production, the different media phases would not induce an increased androgenetic response in breeding lines or increased cultivar response to anther cultures.

Table 13a Summary of analysis from regression analysis for variable embryoid frequency

Source	DF	Sum of squares	Mean square	F value
Regression	1	338	337.7	2.55
Residual	433	57420	132.6	
Corrected Total	434	57758	133.1	

Table 13b Predictions from regression model between entries for variable embryoid frequency

Entries	Embryoid frequency LSMEAN	Standard error LSMEAN	i/j	t-Value 1	2
Atzimba	1.28	0.23	1		
87.2002/3	2.10	0.50	2	1.473204	

P = 0.01 t = 2.6175; P = 0.05 t = 1.95996

Table 13c Predictions from regression model between media treatments for variable embryoid frequency

Media	Embryoid frequency LSMEAN	Standard error LSMEAN	i/j	t-Value 1	2	3
liquid	2.50	0.55	1			
double layer	0.13	0.10	2	4.267893		
standard	2.16	0.41	3	0.5068960	4.860999	

P = 0.01 t = 2.6175; P = 0.05 t = 1.95996

Table 13d Predictions from regression model for entry/media interaction for variable embryoid frequency

Entries	Media	Embryoid frequency LSMEAN	Standard error LSMEAN	I/J	t-Value 1	2	3
Atzimba	liquid	3.85	0.80	1			
Atzimba	double	0.00017	0.00017	2	4.792927		
Atzimba	standard	1.16	0.33	3	3.102260	3.507306	
87.2002/3	liquid	0.00017	0.0046	1			
87.2002/3	double	0.58	0.44	2	1.325378		
87.2002/3	standard	5.06	1.18	3	4.296026	3.566227	

P = 0.01 t = 2.6175; P = 0.05 t = 1.95996

Table 14a Summary of analysis from regression analysis for variable anther response

Source	DF	Sum of squares	Mean square	F value
Regression	1	557	556.70	18.72
Residual	433	12875	29.73	
Corrected Total	434	13432	30.95	

Table 14b Predictions from regression model between entries for variable anther response

Entries	Anther Response LSMEAN	Standard error LSMEAN	i/j	t-Value 1	2
Atzimba	0.26	0.05	1		
87.2002/3	0.82	0.15	2	3.673302	

P = 0.01 t = 2.6175; P = 0.05 t = 1.95996

Table 14c Predictions from regression model between media treatments for variable anther respons

Media	Anther Response LSMEAN	Standard error LSMEAN	i/j	t-Value 1	2	3
liquid	0.27	0.08	1			
double layer	0.07	0.03	2	2.303757		
standard	0.80	0.12	3	3.7705710	6.080219	

P = 0.01 t = 2.6175; P = 0.05 t = 1.95996

Table 14d Predictions from regression model for entry/media interaction for variable anther response

Entries	Media	Anther Response LSMEAN	Standard error LSMEAN	i/j	t-Value 1	2	3
Atzimba	liquid	0.44	0.13	1			
Atzimba	double	0.0005	0.002	2	3.430182		
Atzimba	standard	0.41	0.09	3	0.1957467	4.427399	
87.2002/3	liquid	0.0005	0.004	1			
87.2002/3	double	0.29	0.15	2	1.978890		
87.2002/3	standard	1.93	0.34	3	5.606080	4.384463	

P = 0.01 t = 2.6175; P = 0.05 t = 1.95996

Table 15a Summary of analysis from regression analysis for variable anther productivity

Source	DF	Sum of squares	Mean square	F value
Regression	1	556	556	0.25
Residual	9	19710	2190	
Corrected Total	10	20266	2027	

Table 15b Predictions from regression model between media treatments for variable anther productivity

Media	Anther Productivity LSMEAN	Standard error LSMEAN	i/j	t-Value 1	2	3
liquid	834.8	619.5	1			
double	230.8	373.1	2	0.8352044		
sandard	276.9	71.5	3	0.8946261	0.1213511	

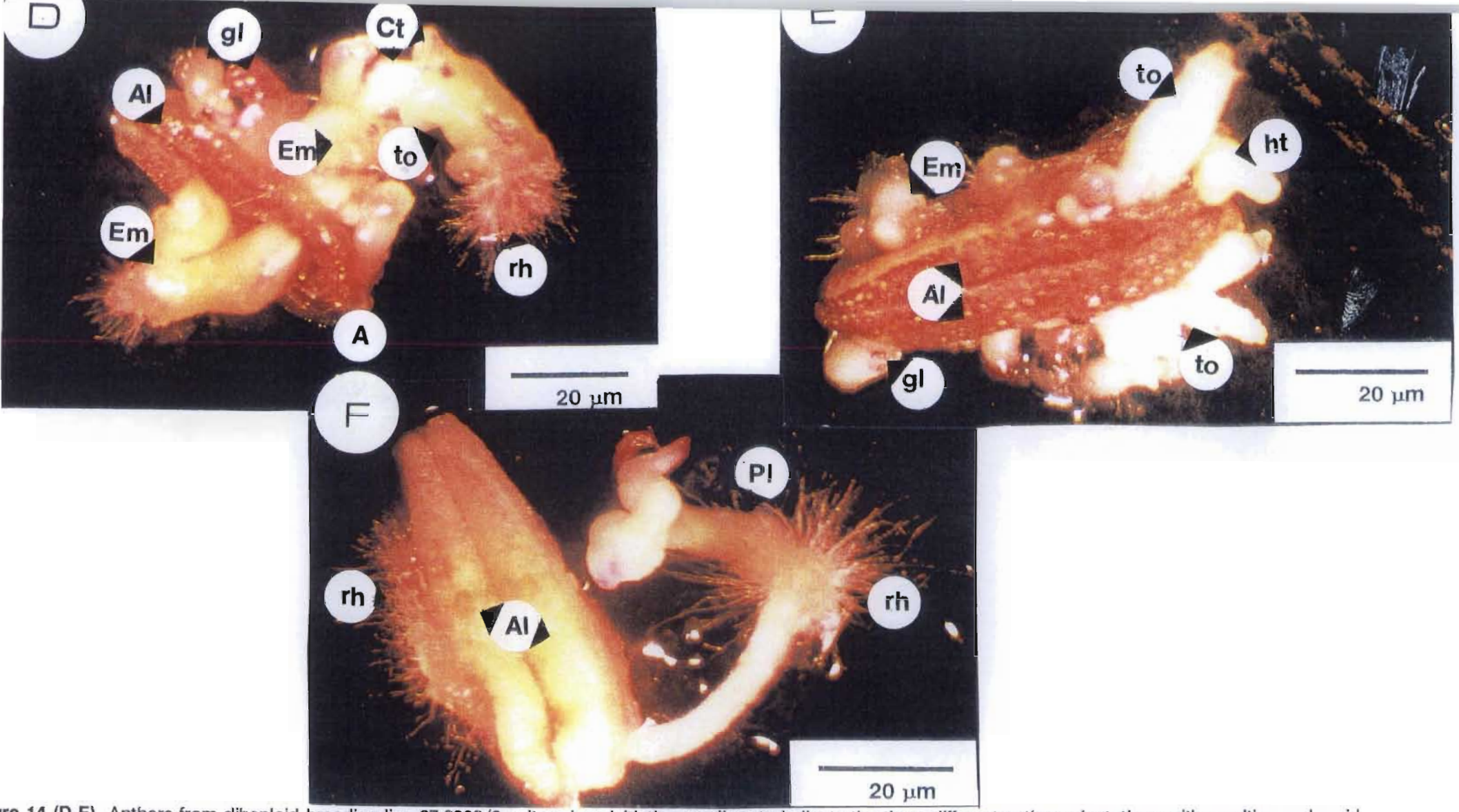
P = 0.01 t = 2.6175; P = 0.05 t = 1.95996

Experiment 2: Anther orientation

This experiment was conducted twice. The first trial commenced in 1993 (trial 1) and the second in 1994 (trial 2). From the material incorporated into the study, the tetraploid cultivar Atzimba and the diploid wild species *S. canasense* showed no response. Only data from dihaploid breeding lines 87.2002/3 and 87.2002/6 were consequently analyzed. Differences in response were investigated between the two trials, the three different anther orientation treatments (lateral, dorsal and ventral) and the trial/orientation interaction.

The differences in the main effect trials were significant and the first trial yielded significantly higher embryoid frequencies (27.50) and anther response (7.52) compared to the second trial (respectively 12.14 and 4.28) (Tables 16c and 17c). Although differences in the main effect entries (cultivars) were not significant in terms of embryoid frequency (Table 16b) or anther response (Table 17b), significance was observed in the correlation of embryoid frequency and anther response, namely anther productivity (Table 18b). From Table 18d it can be seen that of the two responsive breeding lines, 87.2002/3 (119.89) had a significantly higher anther productivity than line 87.2002/6 (78.66).

The differences in the main effect orientations were significant in terms of both embryoid frequency (Table 16d) and anther response (Table 17d). The lateral orientation (Figure 14 D) yielded a significantly higher embryoid frequency (37.09) and anther response (11.42) compared to the dorsal (11.26 embryoid frequency and 2.06 anther response) and ventral orientations (respectively 11.12 and 4.21). The dorsal (Figure 14 E) and ventral (Figure 14 F) orientations did not differ significantly from each other. No significant differences were found in terms of anther productivity. This implies that the different anther orientations have no effect on embryoid production once anthers had already been induced to produce embryoids.



ure 14 (D-F) Anthers from dihaploid breeding line 87.2002/3 cultured on initiation medium to indicate the three different anther orientations with resulting embryoid formation:

D. An anther orientated laterally with a large number of white, microspore derived embryoids visible on the surface of the anther, following the longitudinal rupture of the anther wall. Embryoids at the globular, heart and torpedo shape developmental stages were distinguished, as well as differentiated root-hairs.
E. The dorsal orientation where both sides of the anther wall ruptured for embryoid (globular, heart and torpedo shaped) release.
F. Ventral orientation with an embryoid differentiated into a plantlet. Well differentiated root-hairs were visible.
A, anther; **Al**, anther lobe(s); **Ct**, cotyledons; **Em**, embryoids; **gl**, globular shaped embryoid; **ht**, heart shaped embryoid; **Pl**, plantlet; **rh**, root-hairs; **to**, torpedo shaped embryoid.

The interaction between the two treatment trials and orientation was significant in terms of embryoid frequency (Table 16e). The interaction showed that for the first trial, the lateral orientation (54.02) yielded significantly higher embryoid frequencies compared to both dorsal (15.86) and ventral (12.62) orientations. No significant differences were found between the dorsal and ventral orientations. In the second trial the lateral orientation (20.16) again yielded higher embryoid frequencies than the dorsal orientation (6.65), while no significance was observed between the lateral and ventral (9.62) or between the dorsal and ventral orientations. Thus, for both trials, the lateral orientation yielded the highest embryoid frequency. This trial/orientation interaction was also significant in terms of anther response (Table 17e). The interaction showed that the lateral orientation (16.86) yielded the highest anther response compared to both dorsal (2.36) and ventral orientations (3.33) for the first trial. For the second trial the lateral orientation (5.98) again yielded a significantly higher anther response compared to the dorsal orientation (1.76). No significant differences were found between the lateral and ventral (5.10) orientations. The ventral orientation yielded a significantly higher anther response than the dorsal orientation.

Although the interaction between the treatment trials, entry and orientation was significant in terms of anther productivity, this second order interaction will not be considered here. It did, however, confirm the influence of genetic variation between the two breeding lines.

Table 16a Summary of analysis from regression analysis for variable embryoid frequency

Source	DF	Sum of squares	Mean square	F value	Pr > F
Model	11	90361.05	8214.64	2.58	0.0034
Error	556	1773437.94	3189.64		
Corrected Total	567	1863798.99			

Table 16b Significance of main effects trial, entries, orientation and their interactions

Source	DF	Type III SS	Mean square	F value	Pr > F
Trial	1	19999.65	19999.65	6.27	0.0126
Entries	1	135.49	135.49	0.04	0.8368
Orientation	2	58249.91	29124.96	9.13	0.0001
Trial/Entries	1	833.91	833.91	0.26	0.6093
Trial/Orientation	2	18540.08	9270.04	2.91	0.0555
Trial/Entries/Orientation	4	7217.47	1840.37	0.57	0.6877

Table 16c Predictions from regression model between two trials for variable embryoid frequency

Trial	Embryoid frequency LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN=0	Pr > [T] H0:LSMEAN1=LSMEAN2
1	27.50	5.51	0.0001	0.0126
2	12.14	2.70	0.0001	

Table 16d Predictions from regression model between orientations for variable embryoid frequency

Orientation	Embryoid frequency LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN=0	Pr > [T] H0:LSMEAN(i)=LSMEAN(j)			
				i/j	1	2	3
lateral	37.09	4.84	0.0001	1		0.0014	0.0001
dorsal	11.26	6.44	0.0812	2	0.0014		0.9861
ventral	11.12	4.44	0.0126	3	0.0001	0.9861	

Table 16e Predictions from regression model for trial/orientation interaction for variable embryoid frequency

Trial	Orientation	Embryoid frequency LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN=0	Pr > [T] H0:LSMEAN(i)=LSMEAN(j)						
					i/j	1	2	3	4	5	6
1	lateral	54.02	8.47	0.0001	1		0.0098	0.0003	0.0005	0.0001	0.0001
1	dorsal	15.86	12.04	0.1882	2	0.0098		0.8194	0.7397	0.4748	0.6294
1	ventral	12.62	7.51	0.0934	3	0.0003	0.8194		0.3949	0.4976	0.7351
2	lateral	20.16	4.69	0.0001	4	0.0005	0.7397	0.3949		0.0400	0.1143
2	dorsal	6.65	4.59	0.1485	5	0.0001	0.4748	0.4976	0.0400		0.6532
2	ventral	9.62	4.74	0.0431	6	0.0001	0.6294	0.7351	0.1143	0.6532	

Table 17a Summary of analysis from regression analysis for variable anther response

Source	DF	Sum of squares	Mean square	F value	Pr > F
Model	11	9598.17	872.56	4.75	0.0001
Error	556	102076.98	183.59		
Corrected Total	567	111675.15			

Table 17b Significance of main effects trial, entries, orientation and their interactions

Source	DF	Type III SS	Mean square	F value	Pr > F
Trial	1	886.75	886.75	4.83	0.0284
Entries	1	58.58	58.58	0.32	0.5724
Orientation	2	5643.59	2821.80	15.37	0.0001
Trial/Entries	1	311.84	311.84	1.70	0.1930
Trial/Orientation	2	3138.50	1569.25	8.55	0.0002
Trial/Entries/Orientation	4	885.28	221.32	1.21	0.3074

Table 17c Predictions from regression model between two trials for variable anther response

Trial	Anther Response LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN=0	Pr > [T] H0:LSMEAN1=LSMEAN2
1	7.52	1.32	0.0001	0.0284
2	4.28	0.65	0.0001	

Table 17d Predictions from regression model between orientations for variable anther response

Orientation	Anther Response LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN= 0	Pr > [T] H0:LSMEAN(i)=LSMEAN(j)			
				i/j	1	2	3
lateral	11.42	1.16	0.0001	1		0.0001	0.0001
dorsal	2.06	1.55	0.1824	2	0.0001		0.2527
ventral	4.21	1.07	0.0001	3	0.0001	0.2527	

Table 17e Predictions from regression model for trial/orientation interaction for variable anther response

Trial	Orientation	Anther Response LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN= 0	Pr > [T] H0:LSMEAN(i)=LSMEAN(j)						
					i/j	1	2	3	4	5	6
1	lateral	16.86	2.03	0.0001	1		0.0001	0.0001	0.0001	0.0001	0.0001
1	dorsal	2.36	2.89	0.4136	2	0.0001		0.7777	0.2443	0.8465	0.3781
1	ventral	3.33	1.80	0.0655	3	0.0001	0.7777		0.2124	0.4603	0.4046
2	lateral	5.98	1.12	0.0001	4	0.0001	0.2443	0.2124		0.0077	0.5849
2	dorsal	1.76	1.10	0.1099	5	0.0001	0.8465	0.4603	0.0077		0.0356
2	ventral	5.10	1.14	0.0001	6	0.0001	0.3781	0.4046	0.5849	0.0356	

Table 18a Summary of analysis from regression analysis for variable anther productivity

Source	DF	Sum of squares	Mean square	F value	Pr > F
Model	11	2209944.90	200904.08	5.91	0.0001
Error	556	18897350.01	33988.04		
Corrected Total	567	21107294.92			

Table 18b Significance of main effects trial, entries, orientation and their interactions

Source	DF	Type III SS	Mean square	F value	Pr > F
Trial	1	1563219.61	1563219.61	45.99	0.0001
Entries	1	144051.96	144051.96	4.24	0.0400
Orientation	2	34600.86	17300.43	0.51	0.6014
Trial/Entries	1	106521.39	106521.39	3.13	0.0772
Trial/Orientation	2	39485.93	19742.96	0.58	0.5597
Trial/Entries/Orientation	4	424532.95	106133.24	3.12	0.0148

Table 18c Predictions from regression model between two trials for variable anther productivity

Trial	Anther Productivity LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN=0	Pr > [T] H0:LSMEAN1 = LSMEAN2
1	167.18	17.98	0.0001	0.0001
2	31.37	8.81	0.0004	

Table 18d Predictions from regression model between entries for variable anther productivity

Entries	Anther Productivity LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN=0	Pr > [T] H0:LSMEAN1=LSMEAN2
87.2002/3	119.89	13.57	0.0001	0.0400
87.2002/6	78.66	14.73	0.0001	

Table 18e Predictions from regression model for trial/entry interaction for variable anther productivity

Trial	Entries	Anther Productivity LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN=0	Pr > [T] H0:LSMEAN(i)=LSMEAN(j)				
					i/j	1	2	3	4
1	87.2002/3	205.52	24.40	0.0001	1		0.0335	0.0001	0.0001
1	87.2002/6	128.84	26.43	0.0001	2	0.0335		0.0012	0.0007
2	87.2002/3	34.25	11.89	0.0041	3	0.0001	0.0012		0.7432
2	87.2002/6	28.48	13.01	0.0290	4	0.0001	0.0007	0.7432	

IV DISCUSSION

Experiment 1: Liquid- vs double layered vs standard agar solidified media.

The enhancing effect of liquid medium on the androgenetic response and embryoid production, as observed for tetraploid cultivar Atzimba, correlates with the results of MAHESHWARI, RASHID and TYAGI (1982) and UHRIG (1985). Despite the findings of JOHANSSON (1986) that double layer cultures would, like liquid cultures, increase androgenetic response due to the advantages of the shed-pollen technique, double layer cultures proved of no significance in the present experiments. In contrast with the results from Atzimba, breeding line 87.2002/3 showed the highest androgenetic response and embryoid yield with the utilization of a standard agar solidified medium. Here, the possibility of another determining variable such as anther *orientation* should be kept in mind. Anther orientation proved important in *Datura* and *Nicotiana* (SOPORY and MAHESHWARI, 1976) as well as in *Hordeum* (HUNTER, 1985; SHANNON, NICHOLSON, DUNWELL and DAVIES, 1985; POWELL, BORRINO and GOODALL, 1988). This aspect of controlling anther orientation when using agar solidified media, will be addressed later. It is possible that orientation is of considerable importance, thus leading to higher embryoid yields despite the significant enhancement reported with liquid cultures.

Other factors which could influence the difference in response between Atzimba and 87.2002/3 are the effect of availability and exposure to growth regulators. For instance, in liquid cultures the whole area of the anther is subjected to all medium components, thus reducing competition for important nutrients. In agar solidified cultures, on the other hand, only a part of the anther is in contact with the medium. Due to a possible poorly developed diffusion system, anthers of Atzimba may not have had effective transfer of important nutrients to developing

microspores when cultured on an agar solidified medium. Furthermore, as the plants, tubers and overall appearance of tetraploid potato genotypes are bigger, another possibility could be that Atzimba anthers posses a thicker cuticle, which is less permeable for nutrient absorption. Based on these assumptions of an either underdeveloped diffusion system òr a thick cuticle which hampered the proper diffusion and transfer of essential nutrients, a study can be recommended in which cuticle thickness and permeability are determined. Further, the electronmicroscopic determination of stomatal distribution and frequency on the anther surface may also serve as a profitable field of study.

The possibility exists that Atzimba anthers do not require exposure to light during the culture phase, while 87.2002/3 requires a definite photoperiod for effective embryogenesis. Thus explaining Atzimba's better performance under liquid conditions where cultures were incubated in growth cabinets in the dark (no photoperiod). The difference in response between tetraploid Atzimba and dihaploid line 87.2002/3 may of course be due to genetic factors.

Experiment 2: Anther orientation

Anther orientation proved a determining variable in increasing androgenetic response in, especially responsive genotypes such as the dihaploid breeding lines 87.2002/3 and 87.2002/6.

The results of Tables 5c and 6c indicate that the first trial resulted in an overall higher androgenetic response compared to the second trial. This difference in response between the two trials could be due to various factors such as differences in glasshouse conditions or differences in the physiological state of donor plants. For instance, flowers were not collected from the same plants/tubers for the two trials. In addition anthers used could have been in different stages of microspore development due to selection of either too young or too old flowers.

According to SUNDERLAND (1974); MAHESHWARI, TYAGI and MALHORTA (1980); MAHESWARI, RASHID and TYAGI (1982) and SOPORY and BAJAJ (1987) the uninucleate stage of microspore development results in the highest yield of microspore derived embryoids. According to MAHESHWARI, TYAGI and MALHORTA (1980) and DUNWELL (1985), the anthers of flowers selected at the beginning of the flowering period have a higher androgenetic response compared to those selected at a later stage. It is possible that flowers from the flowering period early in the year could be more responsive than those flowers harvested from plants flowering later in the year (MAHESHWARI, TYAGI and MALHORTA, 1980; DUNWELL, 1985).

From the results it can be seen that, of the two breeding lines investigated, 87.2002/3 was the most responsive to anther culture. The results indicated that, for both trials 1 and 2, the lateral orientation, where only one lobe of the anther was in contact with the culture medium, resulted in significantly higher embryoid frequencies ánd induced more anthers for microspore derived embryoid production. This correlates well with the results obtained by HUNTER (1985) and SHANNON, NICHOLSON, DUNWELL, and DAVIES (1985) in *Hordeum vulgare* L. These authors reported that anthers in the "up" orientation produced higher yields of microspore derived embryoids as well as an increase in the percentage of responding anthers compared to anthers with both locules in contact with the culture medium. These results do, however, differ from the findings of POWELL, BORRINO and GOODALL (1988), who reported that the lateral orientation did not significantly affect the percentage of responding anthers.

As anthers are modified leaves, stomata and a cuticle occur on anther surfaces. The fact that the lateral orientation resulted in the highest androgenetic response could, therefore, be ascribed to either (i) a higher stomata concentration on the "sides" of the anther ór (ii) that the cuticle

was thinner at the sides. Both these possibilities would result in a more efficient diffusion of nutrients when the "side" of an anther lobe is placed in close contact with nutrient medium. The different orientations had no effect, however, on embryoid production once embryoid formation had already been initiated.

From the data of both experiments it can be concluded that the diploid wild species *S. canasense* is androgenetically unresponsive to our *in vitro* culture conditions and procedures. This unresponsiveness could either be of genetic origin or due to various external factors, either single or in combination. For instance, anthers were not necessarily selected in the most favourable period of microspore development (eg. the uninucleate stage), or the initiation medium used in these experiments did not provide in the specific requirements of this wild species for optimal results.

V RECOMMENDATIONS FOR FUTURE STUDIES

When a liquid culture medium is utilized for potato anther culture, the effect of 'Ficoll' in the medium could be investigated. KAO (1981) (quoted from FOROUGHI-WEHR and WENZEL, 1993) reccommended the addition of 'Ficoll' to liquid medium cultures, as it prevented the small developing embryoids/calli from sinking into the nutrient medium and dying due to anaerobiosis. 'Ficoll' addition may improve embryoid regeneration frequency of responsive potato genotypes. With the utilization of agar solidified media, potato anthers should be cultured in the lateral orientation. FOROUGHI-WEHR and WENZEL (1993) reported, however, that the concentration and composition of sugars and the type of gelling agent are the factors with the greatest influence on medium quality. Therfore, attention could be given to studies concidering the effect of the replacement of agar by either gelrite or potato starch as gelling agent.

CHAPTER 4

MEDIUM MANIPULATION

I INTRODUCTION

Tissue culture techniques, especially anther culture, proved to be very advantageous and gave new perspective to modern plant breeding. The reduction of the tetraploid chromosome level (2n=4x=48) of cultivated potato (*Solanum tuberosum* L.) to the dihaploid (2n=2x=24) and eventually the monohaploid level (2n=x=12) is essential for rapid and efficient gene-transfer and consequent crop improvement. This reduction in ploidy level can theoretically be achieved much faster by means of *in vitro* anther culture techniques, compared to the time consuming and complicated method of parthenogenesis. Consequently, reliable anther culture techniques would greatly facilitate breeding programmes (TIAINEN, 1993). However, the cultivated potato shows poor responses to anther culture and amenability remains restricted to the few clones competent to androgenesis. According to TIAINEN (1993) little is known about the physiological systems controlling this phenomenon. It could be due to genetical differences between genotypes or the fact that cultivation methods and culture media are not yet developed to its full potential (JOHANSSON, 1988). Therefore, it is important to try and identify and overcome these constraints in order to develop culture conditions and techniques which would optimize potato breeding and overall crop improvement.

CAI, SZAREJKO, POLOK and MALUSZYNSKI (1992) indicated using barley anther cultures, that the composition of the culture media and subsequent effect of the growth regulator composition in, especially, the initiation

medium are critical factors for successful induction of microspore divisions and embryoid formation. Speculations and assumptions have been made by several researchers concerning the effect, either beneficial or inhibitory, of cytokinins (GUHA and MAHESHWARI, 1964), auxins (NITSCH, 1969; MAHESHWARI, RASHID and TYAGI, 1982), gibberellins (SOPORY and MAHESHWARI, 1976), abscisic acid (BROWN, BROOKS, PEARSON and MATHIAS, 1989) and other growth regulators. TIAINEN (1993) indicated that both the type, concentration and combination of growth regulators included in culture media, significantly affect embryoid and plantlet production in tetraploid potato anther cultures. This correlated with the assumptions made by SKOOG and MILLER (1957), namely that in *Nicotiana tabacum* cultures (a) the neoformation of organs *in vitro* is determined by the auxin/cytokinin ratio contained in the nutrient medium and that (b) the **type** of organogenesis in the cell layers is determined by the different kinds of auxins and cytokinins used.

Although embryoids have been reported to develop even in the absence of growth regulators in the initiation medium of *N. tabacum* cultures (NITSCH, 1969) as well as certain tetraploid potato clones (JOHANSSON, 1986), either auxins or cytokinins are routinely included in culture media. For species such as potato, however, a combination of both auxin and cytokinin are usually recommended (SOPORY and ROGAN, 1976; FOROUGHI-WEHR, WILSON, MIX and GAUL, 1977; SOPORY, JACOBSEN and WENZEL, 1978). The reaction of anthers to media containing hormones varies greatly both between and within species (SUNDERLAND, 1974). IRIKURA (1975) indicated that the concentration of exogenous growth regulators supplied to the culture medium is not the only determining factor for embryoid- and plantlet development from *Solanum* pollen, but that both the interaction between growth regulators and the type of basal medium used, play an important role.

The growth regulators routinely used in the initiation medium of potato anther cultures are the auxin indole-3-acetic acid (IAA) and the cytokinin benzyladenine (BA). MAHESHWARI, TYAGI and MALHORTA (1980) and WENZEL and FOROUGHI-WEHR (1984) found that the addition of cytokinins proved more efficient and critical in *S. tuberosum* cultures, while the effect of auxins was more prominent in *Nicotiana tabacum* and *Lycopersicon esculentum* anther cultures. Differences exist in the literature concerning the specific optimum concentrations of these two growth regulators. WENZEL and FOROUGHI-WEHR (1984) recommended concentrations of 1.0 mg l^{-1}IAA and 1.0 mg l^{-1}BA, while POWELL and UHRIG (1987) supplemented culture media of different diploid potato genotypes with 0.1 mg l^{-1}IAA and 2.5 mg l^{-1}BA. TIAINEN (1993) indicated, however, that an optimum concentration can be determined for each growth regulator. He regarded 0.1 mg l^{-1}IAA and 1.0 mg l^{-1}BA as optimum concentrations for potato anther cultures.

As the addition of auxins to the media of *Solanum tuberosum* cultures proved to be less essential (WENZEL and FOROUGHI-WEHR, 1984) as well as the fact that the effect of auxins on embryogenesis are, in general, much better understood compared to that of cytokinins (TIAINEN, 1993), this study focused mainly on the effect of different concentrations and concentration-combinations of the auxin IAA and cytokinin BA, on the androgenetic response. The effect of another growth regulator, gibberellic acid (GA_3), was investigated. According to both NITSCH (1969) and TIAINEN (1993), this rarely used growth regulator plays no positive role in promoting embryoid- and plantlet production in both potato and tobacco anther cultures.

In this study two experiments were conducted to determine the effect of different growth regulators on androgenesis and embryogenesis. The first experiment investigated the effect of different auxin/cytokinin concentrations, and various combinations of these concentrations, on the

androgenetic response of different potato genotypes. The second experiment was conducted to attempt direct embryoid production from microspores of tetraploid potato cultivars by altering the time of incubation on the initiation medium. Both the initial culture period of the anthers as well as the effect of media containing GA_3 and media without any growth regulators, were investigated.

II MATERIALS AND METHODS

1. Plant Material

The genotypes incorporated into this study included local tetraploid potato cultivars, a dihaploid breeding line with high androgenetic response and a diploid wild species with no reported androgenetic response. For the first experiment the tetraploid cultivar Atzimba, the dihaploid breeding line 87.2002/3 and the diploid wild species *Solanum canasense* were used. Line 87.2002/3 was obtained from the Max Planck Institute for Plant Breeding, Köln. *S. canasense* was propagated *in vitro* from virus free seed obtained from CIP, Peru. Tuberization of the latter occurred under glass house conditions. For the second experiment the tetraploid cultivars Atzimba, Buffelspoort and BP_1 were selected from the local conventional potato breeding program.

Virus free potato tubers, from each of the selected genotypes, were grown in a glass house under controlled conditions. A day/night temperature of 21°C/14°C, a light intensity of 200 µmol $m^{-2}s^{-1}$ and a 16 hour photoperiod proved essential for optimum flower formation. This light intensity could vary greatly in local glass houses.

2. Anther Culture

2.1. Culture Procedure and Medium Composition

For maximum response in anther culture, anthers have to be in the uninucleate stage of microspore development, that is free of starch (WENZEL and FOROUGHI-WEHR, 1984). Flower buds between the length-range of 4.2 - 4.5 mm (BRINK, DE BRUYN, VAN RHYN and FERREIRA, 1989) were selected for all genotypes and received a cold pretreatment of 6 - 9°C for at least 48 h in the dark prior to culture (WENZEL and FOROUGHI-WEHR, 1984). The optimal flower bud lengths vary between different potato genotypes and can be pre-determined by means of fluorescent techniques as described in Chapter 2. The flower buds were surface sterilized in 70% ethanol for two to three minutes, followed by 10 minutes in 1% sodium hypochlorite with 0.1% Tween-20 added and subsequently washed three times in sterile, distilled water. The anthers were dissected under aseptic conditions from the flower buds and plated on the appropriate initiation medium in 90x10mm sterile, plastic petri-dishes. Before incubation the petri-dishes were sealed with a double layer of parafilm.

The standard initiation medium consisted of the basal medium of LINSMAIER and SKOOG (1965) at half strength, supplemented with filter-sterilized growth regulators as indicated in Table 19[a]. This medium served as initiation medium for the second experiment. This basic formulation was also used for the first experiment, but different concentrations of IAA and BA were included (Tables 20 - 23) to study the effect of differential auxin/cytokinin treatments. Developed embryoids were transferred to an embryoid-regeneration medium (Table 19[b]). As soon as root or shoot formation was observed, embryoids were transferred to an embryoid maturation medium (Table 19[c]). After sufficient development, plantlets were transferred to test tubes containing a modified MURASHIGE and SKOOG medium (1962) (referred to as Osborne medium). After reaching a height

of about 8 cm, rooted plantlets were transferred to sterile soil in pots under glass house conditions. The ploidy of developed plantlets was determined by counting the number of chloroplasts in stomatal guard cells (FRANDSEN, 1968 as quoted by MEYER, SALAMINI and UHRIG, 1993). In the event of callus formation from microspores rather than embryoid production, calli were transferred from anthers to a shoot regeneration medium (Table 19[d]). Regenerated plantlets were transferred to Osborne medium in test tubes and eventually rooted plantlets were established in soil.

Table 19 **Composition of initiation, regeneration and maturation media used in potato anther culture experiments.**

Composition	Media used [a]	[b]	[c]	[d]	Osborne
LS	half strength	half strength	half strength	full strength	full strength
Thiamine HCl	0.2 mg l^{-1}	0.2 mg l^{-1}	0.2 mg l^{-1}	-	-
(myo)-Inositol	50 mg l^{-1}	50 mg l^{-1}	50 mg l^{-1}	-	-
Benzyladenine (BA)	1.0 mg l^{-1}	-	-	-	-
Indole-3-acetic acid (IAA)	0.1 mg l^{-1}	-	-	-	-
Asparagine	7.5 mg l^{-1}	-	-	-	-
Glutamine	7.0 mg l^{-1}	-	-	-	-
Gibberellic acid (GA_3)	-	0.1 mg l^{-1}	-	0.02 mg l^{-1}	0.1 mg l^{-1}
Naphtalene-1-acetic acid (NAA)	-	-	-	0.02 mg l^{-1}	0.01 mg1^{-1}
Zeatin	-	-	-	2.0 mg l^{-1}	-
Glycine	-	-	-	2.0 mg l^{-1}	-
Activated charcoal	5 g l^{-1}	-	-	-	-
Agar	8 g l^{-1}	8 g l^{-1}	8 g l^{-1}	8 g l^{-1}	7 g l^{-1}
Sucrose	60 g l^{-1}	20 g l^{-1}	20 g l^{-1}	20 g l^{-1}	30 g l^{-1}
pH	5.8	5.8	5.8	5.8	5.5

LS = basal medium of LINSMAIER and SKOOG (1965)
[a] = initiation medium;
[b] = embryoid regeneration medium
[c] = embryoid maturation medium;
[d] = shoot regeneration medium

Experiment 1: The effect of differential auxin and cytokinin treatments on the androgenetic response of various potato genotypes

Anthers of Atzimba, *S. canasense* and 87.2002/3, respectively, were cultured on the standard initiation medium [a], containing different combinations of auxin and cytokinin concentrations. For logistic reasons the five anthers from each flower bud were cultured together in a petri-dish. The anthers were transferred to fresh medium every four weeks. Each petri-dish was considered as one replication and 25 anthers (five petri-dishes) were cultured for each differential medium type (treatment) on one day (i.e. 16 treatments x 5 anthers x 5 petri-dishes = 400 anthers of each cultivar/line on one day = 1 replication). Material remained in culture for a period of three months.

This experiment was repeated twice. Table 20 indicates the different auxin and cytokinin concentrations used in the first trial. These concentrations were combined to create a differential treatment consisting of 16 different "types" of initiation media, indicated in Table 21. Due to the possibility that the auxin/cytokinin concentrations used in the first replication could be too high, it was decided to repeat the experiment. This time the initiation medium was supplemented with lower IAA and BA concentrations, as indicated in Table 22. Table 23 represents the 16 differential treatments, resulting from the adapted auxin/cytokinin concentrations, as used in the second trial.

Table 20 Growth regulator concentrations used in Trial 1

[IAA]	0.0 mg l^{-1}	0.1 mg l^{-1}	1.0 mg l^{-1}	1.5 mg l^{-1}
[BA]	0.0 mg l^{-1}	0.5 mg l^{-1}	1.0 mg l^{-1}	1.5 mg l^{-1}

Table 21 Different combinations of hormone concentrations (differential treatments) for Trial 1

	[IAA]			
[BA]	0.0 mg l^{-1}	0.1 mg l^{-1}	1.0 mg l^{-1}	1.5 mg l^{-1}
0.0 mg l^{-1}	BA1 (C)	BA2	BA3	BA4
0.5 mg l^{-1}	BA5	BA6	BA7	BA8
1.0 mg l^{-1}	BA9	BA10*	BA11	BA12
1.5 mg l^{-1}	BA13	BA14	BA15	BA16

(C) = control treatment

* the "standard initiation medium" routinely used for potato anther culture.

Table 22 Growth regulator concentrations used in Trial 2

[IAA]	0.0 mg l^{-1}	0.01 mg l^{-1}	0.1 mg l^{-1}	1.0 mg l^{-1}
[BA]	0.0 mg l^{-1}	0.01 mg l^{-1}	0.1 mg l^{-1}	1.0 mg l^{-1}

Table 23 Different combinations of hormone concentrations (differential treatments) for Trial 2

	[IAA]			
[BA]	0.0 mg l^{-1}	0.01 mg l^{-1}	0.1 mg l^{-1}	1.0 mg l^{-1}
0.0 mg l^{-1}	BA1 (C)	BA2	BA3	BA4
0.01 mg l^{-1}	BA5	BA6	BA7	BA8
0.1 mg l^{-1}	BA9	BA10	BA11	BA12
1.0 mg l^{-1}	BA13	BA14	BA15*	BA16

(C) = control treatment

* the "standard initiation medium" routinely used for potato anther culture.

Experiment 2: The induction of direct embryogenesis in tetraploid potato cultivars

Anthers of the three tetraploid potato cultivars Atzimba, Buffelspoort and BP_1 were cultured initially for one week on the standard initiation medium (Table 19[a]) to screen for internal contamination. The five anthers from each flower bud were cultured together in a petri-dish. The experiment consisted of two control treatments and four other treatments. For the first control treatment anthers remained on initiation medium (Table 19[a]) and were transferred monthly to fresh initiation medium. Anthers of the second control treatment remained for the duration of the experiment, approximately three months, on initiation medium (Table 19[a]) without monthly transfer to fresh medium. The anthers of the four treatments were divided into two groups. Anthers of one group were transferred to LS (LINSMAIER and SKOOG, 1965) medium, that is initiation medium (Table 19[a]) without any growth regulators and hormones, while the same number of anthers were transferred from the second group to an embryoid regeneration medium (Table 19[b]). Anthers were transferred from initiation medium [a] after respectively 7, 14, 21 and 28 days to the two mentioned media groups.

2.2 Statistical Analysis

Complete randomised designs were used in both experiments. Results were recorded every four weeks. The number of responding anthers as well as the total number of embryoids produced were taken into account. From these data the variables embryoid frequency (amount of embryoids formed in 100 anthers), anther response (% anthers that formed embryoids) and anther productivity (number of embryoids produced from responding anthers) were determined. For both experiments, data was log-transformed and analyzed with the general linear model (GLM), using the Poisson distribution. For all three variables investigated (embryoid frequency, anther response and anther productivity), all differences reported were significant ($P < 0.05$), unless otherwise indicated.

III RESULTS

Experiment 1: The effect of differential auxin and cytokinin treatments on the androgenetic response of various potato genotypes

This experiment was conducted twice. The first trial commenced early in 1994 and the second in October 1994. From the material incorporated into the study, the diploid wild species *S. canasense* and tetraploid cultivar Atzimba showed no response and data was analyzed only for the dihaploid breeding line 87.2002/3. The difference in response between the four different BA concentrations, the four IAA concentrations and the BA/IAA interaction were analyzed.

TRIAL 1

According to Table 24b a BA concentration of 1.5 mg l^{-1} yielded a significantly higher embryoid frequency (22.77) compared to BA concentrations of, respectively, 0.0 mg l^{-1} (10.22), 0.5 mg l^{-1} (5.06) and 1.0 mg l^{-1} (7.33), while

the BA concentrations 0.0 mg l^{-1}, 0.5 mg l^{-1} and 1.0 mg l^{-1} did not differ significantly from each other. In terms of anther response, a concentration of 1.5 mg l^{-1} proved significantly better (7.88) than 0.5 mg l^{-1}BA (3.22). No significant differences were found between the other BA concentrations in terms of anther response. This BA concentration of 1.5 mg l^{-1} also yielded a significantly higher anther productivity (258.1) (Table 26b) compared to 0.5 mg l^{-1} (146.2) and 1.0 mg l^{-1} BA (149.0). No significant differences were found between the four different IAA concentrations in terms of embryoid frequency (Table 24c), anther response (Table 25c) or anther productivity (Table 26c). This implies that the addition of IAA to the nutrient medium does not have an effect on either the androgenetic response or embryoid production.

Although the differences were significant in the two main effects IAA concentrations and BA concentrations, as discussed above, only the interaction is of value and was considered further. In terms of embryoid frequency (Table 24d), anther response (Table 25d) and anther productivity (Table 26d) the interaction between the two main effects (BA treatments and IAA treatments) was significant. From Table 24d it can be seen that, in terms of embryoid frequency, the interaction was not significant between BA1IAA1 (9.60), BA1IAA2 (10.40), BA1IAA3 (13.64) and BA1IAA4 (7.00). At a BA concentration of 0.5 mg l^{-1} (BA2) no significant differences were found between the interaction of the four combinations BA2IAA1 (3.20), BA2IAA2 (8.00), BA2IAA3 (6.32) and BA2IAA4 (2.22). The four BA/IAA combinations at a BA concentration of 1.0 mg l^{-1} (BA3); namely BA3IAA1 (6.67), BA3IAA2 (6.36), BA3IAA3 (9.00) and BA3IAA4 (7.50); also did not differ significantly. At a BA concentration of 1.5 mg l^{-1}, BA4IAA3 (31.67) differed significantly from BA4IAA2 (12.17) ($t = 2.018645$).

In terms of anther response (Table 25d), the interaction between all of the four IAA combinations with either BA1 (0.0 mg l^{-1}), BA2 (0.5 mg l^{-1}), BA3 (1.0 mg l^{-1}) or BA4 (1.5 mg l^{-1}) were not significant.

In terms of anther productivity (Table 26d) BA1IAA3 (433.3) differed significantly from BA1IAA1 (175) (t=2.091824) and BA1IAA4 (86.7) (t=3.026700). At a BA concentration of 0.5 mg l^{-1} (BA2) the interaction of the four different BA/IAA combinations; BA2IAA1 (100), BA2IAA2 (175), BA2IAA3 (200) and BA2IAA4 (100); did not differ significantly. No significance was observed at BA concentrations of 1.0 mg l^{-1} (BA3) and 1.5 mg l^{-1} (BA4).

Table 24a Summary of analysis from regression analysis for variable embryoid frequency

Source	DF	Sum of squares	Mean square	F value
Regression	3	1373	457.59	9.15
Residual	359	17949	50.0	
Corrected Total	362	19322	53.38	

Table 24b Predictions from regression model between four BA concentrations for variable embryoid frequency

[BA] mg l^{-1}	Embryoid frequency LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j) i/j	1	2	3	4
0.0	10.22	2.35	1				
0.5	5.06	1.70	2	1.779046			
1.0	7.33	2.02	3	0.932603	0.859799		
1.5	22.77	3.47	4	2.994604	4.583271	3.845451	

P=0.01 t=2.3261; P=0.05 t=1.960

Table 24c Predictions from regression model between four IAA concentrations for variable embryoid frequency

[IAA] mg l^{-1}	Embryoid frequency LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j) i/j	1	2	3	4
0.0	12.09	2.50	1				
0.1	9.50	2.26	2	0.7685223			
1.0	15.45	2.96	3	0.8672130	1.597685		
1.5	9.03	2.28	4	0.9043760	0.146404	1.718274	

P=0.01 t=2.3261; P=0.05 t=1.960

ıble 24d **Predictions from regression model for BA/IAA interaction for variable embryoid frequency**

BA] mg l^{-1}	[IAA] mg l^{-1}	Embryoid frequency LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j) i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
).0	0.0	9.60	4.4	1																
).0	0.1	10.40	4.52	2	0.126824															
).0	1.0	13.64	5.59	3	0.567899	0.450703														
).0	1.5	7.00	4.2	4	0.427437	0.551043	0.949656													
).5	0.0	3.20	2.54	5	1.259716	1.388678	1.700323	0.774196												
).5	0.1	8.00	4.02	6	0.268461	0.396758	0.819127	0.172004	1.009420											
).5	1.0	6.32	4.1	7	0.545381	0.668579	1.055912	0.115855	0.646897	0.414168										
).5	1.5	2.22	2.5	8	1.458316	1.583642	1.864925	0.977957	0.274977	1.351623	0.853796									
1.0	0.0	6.67	3.71	9	0.509091	0.637868	1.038885	0.058887	0.771765	0.243129	0.063298	0.994699								
1.0	0.1	6.36	3.82	10	0.556045	0.682662	1.075243	0.112729	0.688847	0.295734	0.007138	0.906831	0.058215							
1.0	1.0	9.00	4.77	11	0.092458	0.213044	0.631418	0.314686	1.073255	0.160306	0.426079	1.258951	0.385574	0.432002						
1.0	1.5	7.50	3.97	12	0.354353	0.482054	0.895525	0.086515	0.912367	0.088497	0.206760	1.125421	0.152751	0.206920	0.241704					
1.5	0.0	28.33	7.71	13	2.109907	2.006209	1.542541	2.429453	3.095736	2.338103	2.520510	3.221393	2.531504	2.553331	2.132082	2.401961				
1.5	0.1	12.17	5.17	14	0.378560	0.257745	0.193059	0.776160	1.557224	0.636739	0.886578	1.732627	0.864316	0.903835	0.450647	0.716431	1.740828			
1.5	1.0	31.67	8.16	15	2.380622	2.280174	1.822852	2.688110	3.331313	2.602105	2.775916	3.450750	2.788996	2.809137	2.398458	2.663508	0.297516	2.018645		
1.5	1.5	18.26	6.32	16	1.124558	1.011584	0.547559	1.483862	2.211027	1.369793	1.584938	2.360039	1.611425	1.169482	1.169482	1.441690	1.010103	0.745844	1.299261	

=0.01 t=2.6175; P=0.05 t=1.95996

= BA1IAA1 = BA(0.0 mg l^{-1});IAA(0.0 mg l^{-1})
= BA1IAA2 = BA(0.0 mg l^{-1});IAA(0.1 mg l^{-1})
= BA1IAA3 = BA(0.0 mg l^{-1});IAA(1.0 mg l^{-1})
= BA1IAA4 = BA(0.0 mg l^{-1});IAA(1.5 mg l^{-1})
= BA2IAA1 = BA(0.5 mg l^{-1});IAA(0.0 mg l^{-1})
= BA2IAA2 = BA(0.5 mg l^{-1});IAA(0.1 mg l^{-1})
= BA2IAA3 = BA(0.5 mg l^{-1});IAA(1.0 mg l^{-1})
= BA2IAA4 = BA(0.5 mg l^{-1});IAA(1.5 mg l^{-1})
= BA3IAA1 = BA(1.0 mg l^{-1});IAA(0.0 mg l^{-1})
) = BA3IAA2 = BA(1.0 mg l^{-1});IAA(0.1 mg l^{-1})
= BA3IAA3 = BA(1.0 mg l^{-1});IAA(1.0 mg l^{-1})
2 = BA3IAA4 = BA(1.0 mg l^{-1});IAA(1.5 mg l^{-1})
= BA4IAA1 = BA(1.5 mg l^{-1});IAA(0.0 mg l^{-1})
= BA4IAA2 = BA(1.5 mg l^{-1});IAA(0.1 mg l^{-1})
= BA4IAA3 = BA(1.5 mg l^{-1});IAA(1.0 mg l^{-1})
= BA4IAA4 = BA(1.5 mg l^{-1});IAA(1.5 mg l^{-1})

Table 25a Summary of analysis from regression analysis for variable anther response

Source	DF	Sum of squares	Mean square	F value
Regression	3	188	62.75	2.96
Residual	359	7613	21.21	
Corrected Total	362	7801	21.55	

Table 25b Predictions from regression model between four BA concentrations for anther response

[BA] mg l^{-1}	Anther Response LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j) i/j	1	2	3	4
0.0	5.66	1.12	1				
0.5	3.22	0.88	2	1.709057			
1.0	4.89	1.07	3	0.495809	1.208805		
1.5	7.88	1.31	4	1.285396	2.946307	1.766808	

P=0.01 t=2.3261; P=0.05 t=1.960

Table 25c Predictions from regression model between four IAA concentrations for variable anther response

[IAA] mg l^{-1}	Anther Response LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j) i/j	1	2	3	4
0.0	5.37	1.07	1				
0.1	4.49	0.99	2	0.6043234			
1.0	6.47	1.25	3	0.5476831	0.0118807		
1.5	5.58	1.16	4	0.1323118	0.7138217	0.6504144	

P=0.01 t=2.3261; P=0.05 t=1.960

Table 25d **Predictions from regression model for BA/IAA interaction for variable anther response**

$[BA]_t$ mg l^{-1}	$[IAA]_t$ mg l^{-1}	Anther Response LSMEAN	Standard error LSMEAN	Pr > |T| H0:LSMEAN(i)=LSMEAN(j) i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
0.0	0.0	4.80	2.01	1																
0.0	0.1	4.03	1.76	2	0.288212															
0.0	1.0	4.55	2.07	3	0.086646	0.191382														
0.0	1.5	10.00	3.25	4	1.360781	1.615278	1.414397													
0.5	0.0	3.20	1.65	5	0.615266	0.344043	0.509983	1.865641												
0.5	0.1	4.00	1.84	6	0.293577	0.011782	0.198587	1.606548	0.323696											
0.5	1.0	3.16	1.88	7	0.595891	0.337829	0.497085	1.821773	0.015991	0.434314										
0.5	1.5	2.22	1.61	8	1.001823	0.758812	0.888480	2.145067	0.425100	0.849620	0.379771									
1.0	0.0	5.00	2.07	9	0.069317	0.357002	0.153719	1.297612	0.679977	0.361067	0.658012	1.060096								
1.0	0.1	3.64	1.88	10	0.421484	0.151441	0.325430	1.693929	0.175910	0.136852	0.180538	0.573696	0.486357							
1.0	1.0	6.00	2.53	11	0.371373	0.639203	0.443572	0.971189	0.927000	0.639317	0.901006	1.260490	0.305912	0.748724						
1.0	1.5	5.00	2.10	12	0.068802	0.354015	0.152609	1.292179	0.673987	0.358159	0.652811	1.050584	0	0.482512	0.304137					
1.5	0.0	8.34	2.67	13	1.059244	1.347764	1.121822	0.394564	1.637624	1.338429	1.586295	1.962890	0.988624	1.439302	0.636166	0.983251				
1.5	0.1	6.09	2.36	14	0.416136	0.699726	0.490573	0.973490	1.003611	0.698407	0.971072	1.354630	0.347224	0.811989	0.026013	0.345040	0.631403			
1.5	1.0	11.67	3.20	15	1.817988	2.091965	1.868200	0.366150	2.352551	2.077866	2.292943	2.638050	1.750126	2.163611	1.389936	1.742637	0.799021	1.403375		
1.5	1.5	5.22	2.16	16	0.142347	0.427097	0.223950	1.224912	0.743165	0.429962	0.719383	1.113581	0.073536	0.551760	0.234471	0.073027	0.908479	0.271939	1.670643	

P=0.01 t=2.6175; P=0.05 t=1.95996

= BA1IAA1 = BA(0.0 mg l^{-1});IAA(0.0 mg l^{-1})
= BA1IAA2 = BA(0.0 mg l^{-1});IAA(0.1 mg l^{-1})
= BA1IAA3 = BA(0.0 mg l^{-1});IAA(1.0 mg l^{-1})
= BA1IAA4 = BA(0.0 mg l^{-1});IAA(1.5 mg l^{-1})
= BA2IAA1 = BA(0.5 mg l^{-1});IAA(0.0 mg l^{-1})
= BA2IAA2 = BA(0.5 mg l^{-1});IAA(0.1 mg l^{-1})
= BA2IAA3 = BA(0.5 mg l^{-1});IAA(1.0 mg l^{-1})
= BA2IAA4 = BA(0.5 mg l^{-1});IAA(1.5 mg l^{-1})
= BA3IAA1 = BA(1.0 mg l^{-1});IAA(0.0 mg l^{-1})
0= BA3IAA2 = BA(1.0 mg l^{-1});IAA(0.1 mg l^{-1})
1= BA3IAA3 = BA(1.0 mg l^{-1});IAA(1.0 mg l^{-1})
2= BA3IAA4 = BA(1.0 mg l^{-1});IAA(1.5 mg l^{-1})
3= BA4IAA1 = BA(1.5 mg l^{-1});IAA(0.0 mg l^{-1})
4= BA4IAA2 = BA(1.5 mg l^{-1});IAA(0.1 mg l^{-1})
5= BA4IAA3 = BA(1.5 mg l^{-1});IAA(1.0 mg l^{-1})
6= BA4IAA4 = BA(1.5 mg l^{-1});IAA(1.5 mg l^{-1})

Table 26a Summary of analysis from regression analysis for variable anther productivity

Source	DF	Sum of squares	Mean square	F value
Regression	3	708	235.97	2.62
Residual	57	5142	90.21	
Corrected Total	60	5850	97.50	

Table 26b Predictions from regression model between four BA concentrations for anther productivity

[BA] mg l^{-1}	Anther Productivity LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i) = LSMEAN(j) i/j	1	2	3	4
0.0	207.7	36.5	1				
0.5	146.2	31.8	2	1.270409			
1.0	149.0	29.0	3	1.259167	0.065059		
1.5	258.1	36.0	4	0.983099	2.329613	2.360057	

P = 0.01 t = 2.3261; P = 0.05 t = 1.960

Table 26c Predictions from regression model between four IAA concentrations for variable anther productivity

[IAA] mg l^{-1}	Anther Productivity LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i) = LSMEAN(j) i/j	1	2	3	4
0.0	180.4	33.1	1				
0.1	189.6	35.8	2	0.188691			
1.0	236.9	34.4	3	1.183530	0.9526924		
1.5	156.6	32.1	4	0.516171	0.6863021	1.706669	

P = 0.01 t = 2.3261; P = 0.05 t = 1.960

Table 26d Predictions from regression model for BA/IAA interaction for variable anther productivity

[BA] mg l^{-1}	[IAA] mg l^{-1}	Anther Productivity LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j) i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
0.0	0.0	175	59.5	1																
0.0	0.1	237.5	98.1	2	0.544739															
0.0	1.0	433.3	108.2	3	2.091824	1.340629														
0.0	1.5	86.7	37.5	4	1.255485	1.435874	3.026700													
0.5	0.0	100	45	5	1.005354	1.273989	2.844229	0.227052												
0.5	0.1	175	59.5	6	0	0.544739	2.091824	1.255485	1.005354											
0.5	1.0	200	73.5	7	0.264369	0.305923	1.783595	1.373106	1.160342	0.420164										
0.5	1.5	100	63.7	8	0.860426	1.175544	2.654540	0.179928	0	1.260372	1.028148									
1.0	0.0	111.1	54.7	9	0.790618	1.125360	2.657521	0.367913	0.156710	0.790618	0.970306	0.132201								
1.0	0.1	175	59.5	10	0	0.544739	2.091824	1.255485	1.005354	0	0.264369	0.860426	0.790618							
1.0	1.0	150	49.3	11	0.323539	0.796968	2.382631	1.021933	0.749070	0.323539	0.564954	0.620738	0.528259	0.323539						
1.0	1.5	150	55.1	12	0.308284	0.777674	2.333189	0.949733	0.702832	0.308284	0.544307	0.593654	0.501025	0.308284	0					
1.5	0.0	316.7	80.1	13	0.050208	0.625357	0.866125	2.600529	2.358640	1.420109	1.073480	2.117429	2.119689	1.420109	1.772352	1.714641				
1.5	0.1	170.8	58.8	14	0.898575	0.583183	2.131634	1.205905	0.956195	0.050208	0.310223	0.816705	0.743379	0.050208	0.271070	0.258122	1.468320			
1.5	1.0	246.9	53.5	15	1.330228	0.084124	1.544272	2.452025	2.101307	0.898575	0.515899	1.765918	1.774847	0.898575	1.331936	1.261717	0.724640	0.957275		
1.5	1.5	322.2	93.3	16	0.142347	0.625633	0.777624	2.342021	2.145094	1.330228	1.028849	1.966866	1.951872	1.330228	1.631851	1.589214	0.044727	1.372832	0.700135	

P=0.01 t=2.6175; P=0.05 t=1.95996

1 = BA1IAA1 = BA(0.0 mg l^{-1});IAA(0.0 mg l^{-1})
2 = BA1IAA2 = BA(0.0 mg l^{-1});IAA(0.1 mg l^{-1})
3 = BA1IAA3 = BA(0.0 mg l^{-1});IAA(1.0 mg l^{-1})
4 = BA1IAA4 = BA(0.0 mg l^{-1});IAA(1.5 mg l^{-1})
5 = BA2IAA1 = BA(0.5 mg l^{-1});IAA(0.0 mg l^{-1})
6 = BA2IAA2 = BA(0.5 mg l^{-1});IAA(0.1 mg l^{-1})
7 = BA2IAA3 = BA(0.5 mg l^{-1});IAA(1.0 mg l^{-1})
8 = BA2IAA4 = BA(0.5 mg l^{-1});IAA(1.5 mg l^{-1})
9 = BA3IAA1 = BA(1.0 mg l^{-1});IAA(0.0 mg l^{-1})
10 = BA3IAA2 = BA(1.0 mg l^{-1});IAA(0.1 mg l^{-1})
11 = BA3IAA3 = BA(1.0 mg l^{-1});IAA(1.0 mg l^{-1})
12 = BA3IAA4 = BA(1.0 mg l^{-1});IAA(1.5 mg l^{-1})
13 = BA4IAA1 = BA(1.5 mg l^{-1});IAA(0.0 mg l^{-1})
14 = BA4IAA2 = BA(1.5 mg l^{-1});IAA(0.1 mg l^{-1})
15 = BA4IAA3 = BA(1.5 mg l^{-1});IAA(1.0 mg l^{-1})
16 = BA4IAA4 = BA(1.5 mg l^{-1});IAA(1.5 mg l^{-1})

TRIAL 2

Significantly higher embryoid frequencies (Table 27a) were recorded with the BA concentrations 0.01 mg l^{-1} (77.33), 0.1 mg l^{-1} (98.24) and 1.0 mg l^{-1} (92.34) compared to a BA concentration of 0.0 mg l^{-1} (44.13), while the BA concentrations 0.01 mg l^{-1}, 0.1 mg l^{-1} and 1.0 mg l^{-1} did not differ significantly from each other. The observations concerning the effect of BA concentrations in terms of anther response (Table 28b) was similar to that of embryoid frequency. From Table 27c it can be seen that an IAA concentration of 0.1 mgl^{-1} yielded a significantly higher embryoid frequency (100.94) compared to both 0.0 mg l^{-1}IAA (67.16) and 1.0 mg l^{-1}IAA (62.60).

A BA concentration of 0.1 mg l^{-1} had a significantly higher anther productivity (416.5) compared to BA concentrations of 0.0 mg l^{-1} (250.8), 0.01 mg l^{-1} (268.4) and 1.0 mg l^{-1} (301.6) (Table 29b). An IAA concentration of 0.1 mg l^{-1} also yielded significantly greater anther productivity (Table 29c) (372.6) compared to IAA concentrations of, respectively, 0.0 mg l^{-1} (294.8), 0.01 mg l^{-1} (290.5) and 1.0 mg l^{-1} (268.7).

The interaction between the two main effects (BA treatments and IAA treatments) was significant in terms of embryoid frequency (Table 27d), anther response (Table 28d) and anther productivity (Table 29d). Although the differences in the two main effects (as discussed above) were significant, only the interaction is of value and was considered in the analysis. From Table 27d it can be seen that the interaction between the BA/IAA combinations BA1IAA3 (87.69) (t=3.380944) and BA1IAA4 (44.80) (t=2.004442) gave significantly higher embryoid frequencies compared to BA1IAA1 (9.61). No significant differences were found, however, between BA1IAA3 and BA1IAA4. BA1IAA3 also resulted in a higher response compared to BA1IAA2 (30.49) (t=2.189186). At a relatively low BA concentration of 0.01 mg l^{-1} (BA2) no significant differences were found between the interaction of the four combinations BA2IAA1 (73.00), BA2IAA2 (79.09), BA2IAA3 (82.50) and BA2IAA4 (74.17). The interaction of the four BA/IAA combinations at a BA concentration of 0.1 mg l^{-1}

(BA3); BA3IAA1 (87.20), BA3IAA2 (146.32), BA3IAA3 (82.40) and BA3IAA4 (87.27) also did not differ significantly. At a relatively high BA concentration of 1.0 mg l^{-1}, BA4IAA3 (157.14) differed significantly from BA4IAA2 (72.50) (t=2.188016) and from BA4IAA4 (45.83) (t=3.043478).

In terms of anther response (Table 28d) the interaction of the BA/IAA combinations BA1IAA3 (27.69) (t=3.203101) and BA1IAA4 (18.40) (t=2.152221) yielded a significantly higher response compared to BA1IAA1 (5.60). No significant differences were found, however, between BA1IAA3 and BA1IAA4. BA1IAA3 also resulted in a higher anther response compared to BA1IAA2 (11.43) (t=2.109389). At a BA concentration of 0.01 mg l^{-1} (BA2) the interaction between the four BA/IAA combinations was not significant. At the relatively low BA concentration of 0.1 mg l^{-1} (BA3) the interaction between BA3IAA2 yielded a significantly higher anther response (38.95) (t=2.297813) compared to BA3IAA3 (16.00). The interaction between BA4IAA1 (39.20) and BA4IAA4 (16.67) was significant (t=2.459370) at the relatively high BA concentration of 1.0 mg l^{-1} (BA4). The interaction between BA4IAA3 (38.10) and BA4IAA4 differed significantly (t=2.222846).

In terms of anther productivity, the interaction between any of the four IAA combinations with either BA1 (0.0 mg l^{-1}), BA2 (0.01 mg l^{-1}), BA3 (0.1 mg l^{-1}) or BA4 (1.0 mg l^{-1}) were not significant (Table 29d).

Table 27a Summary of analysis from regression analysis for variable embryoid frequency

Source	DF	Sum of squares	Mean square	F value
Regression	3	2370	790.1	5.30
Residual	368	54877	149.1	
Corrected Total	371	57247	154.3	

Table 27b Predictions from regression model between four BA concentrations for variable embryoid frequency

[BA] mg l^{-1}	Embryoid frequency LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j) i/j	1	2	3	4
0.0	44.13	8.20	1				
0.01	77.33	11.32	2	2.375174			
0.1	98.24	12.68	3	3.583346	1.230160		
1.0	92.34	12.10	4	3.298266	0.9058746	0.3366253	

P=0.01 t=2.3261; P=0.05 t=1.960

Table 27c Predictions from regression model between four IAA concentrations for variable embryoid frequency

[IAA] mg l^{-1}	Embryoid frequency LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j) i/j	1	2	3	4
0.0	67.16	10.18	1				
0.01	79.74	11.66	2	0.8127334			
0.1	100.94	12.50	3	2.0954210	1.240200		
1.0	62.60	9.87	4	0.3215982	1.121982	2.407245	

P=0.01 t=2.3261; P=0.05 t=1.960

able 27d Predictions from regression model for BA/IAA interaction for variable embryoid frequency

BA] ng l^{-1}	[IAA] mg l^{-1}	Embryoid ..equency LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j) I/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
.0	0.0	9.61	7.27	1																
.0	0.01	30.49	14.22	2	1.307399															
.0	0.1	87.69	21.92	3	3.380944	2.189186														
.0	1.0	44.80	15.98	4	2.004442	0.668977	1.581111													
.01	0.0	73.00	22.81	5	2.647811	1.581506	0.464357	1.012545												
.01	0.01	79.09	22.64	6	2.921953	1.817819	0.272905	1.237390	0.189494											
.01	0.1	82.50	22.13	7	3.129191	1.977202	0.166622	1.381131	0.298921	0.149200										
.01	1.0	74.17	20.99	8	2.906360	1.722854	0.445483	1.113315	0.037744	0.215546	0.273105									
.1	0.0	87.20	22.27	9	3.312046	2.146257	0.015681	1.546875	0.445439	0.255375	0.149702	0.425778								
.1	0.01	146.32	33.14	10	4.029409	3.211968	1.475583	2.759327	1.822463	1.675091	1.601519	1.839245	1.480680							
.1	0.1	82.40	21.66	11	3.185906	2.003420	0.171663	1.396895	0.298834	0.105641	0.003229	0.272862	0.154509	1.61452						
.1	1.0	87.27	23.78	12	3.123081	2.049276	0.012986	1.482350	0.433064	0.249134	0.146841	0.413007	0.002149	1.44769	0.151403					
.0	0.0	101.60	24.07	13	3.658535	2.543582	0.427272	1.965968	0.862455	0.681204	0.584149	0.858900	0.439131	1.09182	0.592943	0.423517				
.0	0.01	72.50	20.73	14	2.862822	1.671146	0.503483	1.058291	0.016222	0.214679	0.329785	0.056608	0.483154	1.88848	0.330204	0.468188	0.916065			
.0	0.1	157.14	32.66	15	4.409230	3.555447	1.765649	3.089675	2.112114	1.964030	1.891948	2.137114	1.769285	0.23254	1.907135	1.729453	1.368944	2.188016		
.0	1.0	45.83	16.46	16	2.0'2892	0.705230	1.527068	0.044898	2.145094	1.188235	1.329576	1.062451	1.493897	2.71575	1.344261	1.432872	1.912562	1.007553	3.043478	

=0.01 t=2.6175; P=0.05 t=1.95996

= BA1IAA1 = BA(0.0 mg l^{-1});IAA(0.0 mg l^{-1})
= BA1IAA2 = BA(0.0 mg l^{-1});IAA(0.01 mg l^{-1})
= BA1IAA3 = BA(0.0 mg l^{-1});IAA(0.1 mg l^{-1})
= BA1IAA4 = BA(0.0 mg l^{-1});IAA(1.0 mg l^{-1})
= BA2IAA1 = BA(0.01 mg l^{-1});IAA(0.0 mg l^{-1})
= BA2IAA2 = BA(0.01 mg l^{-1});IAA(0.01 mg l^{-1})
= BA2IAA3 = BA(0.01 mg l^{-1});IAA(0.1 mg l^{-1})
= BA2IAA4 = BA(0.01 mg l^{-1});IAA(1.0 mg l^{-1})
= BA3IAA1 = BA(0.1 mg l^{-1});IAA(0.0 mg l^{-1})
= BA3IAA2 = BA(0.1 mg l^{-1});IAA(0.01 mg l^{-1})
= BA3IAA3 = BA(0.1 mg l^{-1});IAA(0.1 mg l^{-1})
= BA3IAA4 = BA(0.1 mg l^{-1});IAA(1.0 mg l^{-1})
= BA4IAA1 = BA(1.0 mg l^{-1});IAA(0.0 mg l^{-1})
= BA4IAA2 = BA(1.0 mg l^{-1});IAA(0.01 mg l^{-1})
= BA4IAA3 = BA(1.0 mg l^{-1});IAA(0.1 mg l^{-1})
= BA4IAA4 = BA(1.0 mg l^{-1});IAA(1.0 mg l^{-1})

Table 28a Summary of analysis from regression analysis for variable anther response

Source	DF	Sum of squares	Mean square	F value
Regression	3	439	146.40	3.79
Residual	368	14223	38.65	
Corrected Total	371	14662	39.52	

Table 28b Predictions from regression model between four BA concentrations for variable anther response

[BA] mg l^{-1}	Anther Response LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j) i/j	1	2	3	4
0.0	16.08	2.53	1				
0.01	28.22	3.48	2	2.821631			
0.1	24.62	3.23	3	2.081454	0.7582174		
1.0	28.94	3.45	4	3.005904	0.1469299	0.9140856	

P=0.01 t=2.3261; P=0.05 t=1.960

Table 28c Predictions from regression model between four IAA concentrations for variable anther response

[IAA] mg l^{-1}	Anther Response LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j) i/j	1	2	3	4
0.0	23.24	3.09	1				
0.01	24.80	3.33	2	0.3434008			
0.1	26.90	3.32	3	0.8069724	0.4465933		
1.0	22.51	3.03	4	0.1686808	0.5086403	0.9766824	

P=0.01 t=2.3261; P=0.05 t=1.960

able 28d Predictions from regression model for BA/IAA interaction for variable anther response

BA] ng l^{-1}	[IAA] mg l^{-1}	Anther Response LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i) = LSMEAN(j) i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
.0	0.0	5.60	2.85	1																
.0	0.01	11.43	4.47	2	1.099738															
.0	0.1	27.69	6.28	3	3.203101	2.109389														
.0	1.0	18.40	5.22	4	2.152221	1.014208	1.137616													
.01	0.0	29.00	7.33	5	2.975371	2.046488	0.135719	1.177943												
.01	0.01	29.09	7.00	6	3.107989	2.126308	0.148870	1.224228	0.008880											
.01	0.1	26.67	6.42	7	2.999645	1.948134	0.113576	0.999475	0.239122	0.317767										
.01	1.0	26.33	6.62	8	3.153694	2.115721	0.070138	1.177870	0.067835	0.098989	0.180009									
.1	0.0	20.00	5.44	9	2.344764	1.217171	0.925553	0.212220	0.985965	1.025346	0.792645	0.972173								
.1	0.01	38.95	8.72	10	3.635303	2.808466	1.047830	2.022038	0.873455	0.881770	1.134052	0.970024	1.843790							
.1	0.1	16.00	4.87	11	1.643108	0.691332	1.470990	0.336182	1.477217	1.535048	1.324130	1.500301	0.547840	2.297813						
.1	1.0	27.27	6.78	12	2.946435	1.950519	0.045447	1.036617	0.173263	0.186759	0.064259	0.111863	0.836340	1.057428	1.350061					
.0	0.0	39.20	7.63	13	4.125281	3.140354	1.164736	2.249927	0.964043	0.976382	1.256566	1.076073	2.048934	0.021576	2.563036	1.168792				
.0	0.01	22.50	5.90	14	2.579251	1.495524	0.602315	0.520456	0.690790	0.719843	0.478249	0.657449	0.311519	1.562432	0.849641	0.530727	1.731459			
.0	0.1	38.10	8.20	15	3.743740	2.855701	1.007888	2.026642	0.827379	0.835693	1.097536	0.927059	1.839355	0.071012	2.317258	1.017863	0.098208	1.544252		
.0	1.0	16.67	5.07	16	1.903327	0.775248	1.365359	0.237739	1.383440	1.436968	1.222413	1.398346	0.447804	2.208830	0.095305	1.252066	2.459370	0.749441	2.222646	

0.01 t=2.6175; P=0.05 t=1.95996

= BA1IAA1 = BA(0.0 mg l^{-1});IAA(0.0 mg l^{-1})
= BA1IAA2 = BA(0.0 mg l^{-1});IAA(0.01 mg l^{-1})
= BA1IAA3 = BA(0.0 mg l^{-1});IAA(0.1 mg l^{-1})
= BA1IAA4 = BA(0.0 mg l^{-1});IAA(1.0 mg l^{-1})
= BA2IAA1 = BA(0.01 mg l^{-1});IAA(0.0 mg l^{-1})
= BA2IAA2 = BA(0.01 mg l^{-1});IAA(0.01 mg l^{-1})
= BA2IAA3 = BA(0.01 mg l^{-1});IAA(0.1 mg l^{-1})
= BA2IAA4 = BA(0.01 mg l^{-1});IAA(1.0 mg l^{-1})
= BA3IAA1 = BA(0.1 mg l^{-1});IAA(0.0 mg l^{-1})
= BA3IAA2 = BA(0.1 mg l^{-1});IAA(0.01 mg l^{-1})
= BA3IAA3 = BA(0.1 mg l^{-1});IAA(0.1 mg l^{-1})
= BA3IAA4 = BA(0.1 mg l^{-1});IAA(1.0 mg l^{-1})
= BA4IAA1 = BA(1.0 mg l^{-1});IAA(0.0 mg l^{-1})
= BA4IAA2 = BA(1.0 mg l^{-1});IAA(0.01 mg l^{-1})
= BA4IAA3 = BA(1.0 mg l^{-1});IAA(0.1 mg l^{-1})
= BA4IAA4 = BA(1.0 mg l^{-1});IAA(1.0 mg l^{-1})

Table 29a Summary of analysis from regression analysis for variable anther productivity

Source	DF	Sum of squares	Mean square	F value
Regression	3	2345	781.6	7.54
Residual	190	19707	103.7	
Corrected Total	193	22051	114.3	

Table 29b Predictions from regression model between four BA concentrations for anther productivity

[BA] mg l^{-1}	Anther Productivity LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j)			
			i/j	1	2	3	4
0.0	250.8	26.2	1				
0.01	268.4	22.5	2	0.5096228			
0.1	416.5	30.3	3	4.136648	3.924174		
1.0	301.6	24.1	4	1.427028	1.006958	2.967794	

P=0.01 t=2.3261; P=0.05 t=1.960

Table 29c Predictions from regression model between four IAA concentrations for variable anther productivity

[IAA] mg l^{-1}	Anther Productivity LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j)			
			i/j	1	2	3	4
0.0	294.8	25.9	1				
0.01	290.5	25.3	2	0.1187637			
0.1	372.6	26.0	3	2.119954	2.263082		
1.0	268.7	24.1	4	0.7377416	0.6239021	2.930763	

P=0.01 t=2.3261; P=0.05 t=1.960

able 29d Predictions from regression model for BA/IAA interaction for variable anther productivity

BA] mg l^{-1}	[IAA] mg l^{-1}	Anther Productivity LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j) i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
.0	0.0	191.7	70.3	1																
.0	0.01	251.7	27.1	2	0.796363															
.0	0.1	275.2	40.9	3	1.026656	0.478972														
.0	1.0	235.4	45.0	4	0.523547	0.310298	0.654502													
.01	0.0	276.3	53.4	5	0.958297	0.410801	0.016354	0.585688												
.01	0.01	246.9	39.9	6	0.682883	0.099517	0.495287	0.191216	0.441043											
.01	0.1	299.8	45.4	7	1.291742	0.909725	0.402578	1.007461	0.335279	1.325120										
.01	1.0	253.6	43.2	8	0.750188	0.037257	0.363087	0.291761	0.330488	0.167890	0.737207									
.1	0.0	409.7	62.0	9	2.325727	2.335069	1.810832	2.275178	1.630281	2.208077	1.430151	2.065740								
.1	0.01	380.4	57.2	10	2.082075	2.033338	1.496056	1.992322	1.330315	1.914217	1.103695	1.768963	0.347340							
.1	0.1	556.3	69.2	11	3.696102	4.098646	3.497002	3.887587	3.203359	3.873360	3.099192	3.710584	1.577837	1.959230						
.1	1.0	318.9	52.4	12	1.450724	1.139119	0.657417	1.208906	0.569403	1.093199	0.275486	0.961543	1.118539	0.792800	2.734994					
.0	0.0	279.4	39.0	13	1.090886	0.583267	0.074318	0.738896	0.046881	0.582496	0.340846	0.443299	1.778933	1.458898	3.485946	0.604711				
.0	0.01	294.9	50.4	14	1.193064	0.754930	0.303509	0.880621	0.253308	0.746710	0.072236	0.622169	1.436779	1.121510	3.053438	0.330104	0.243224			
.0	0.1	365.5	51.9	15	1.988957	1.943661	1.366548	1.893958	1.197864	1.811665	0.952798	1.657123	0.546654	0.192914	2.205780	0.631847	1.326247	0.975882		
.0	1.0	258.3	54.4	16	0.749240	0.108595	0.248310	0.324363	0.236129	0.168979	0.585698	0.067659	1.835541	1.546785	3.385489	0.802306	0.315229	0.493536	1.425797	

0.01 t=2.6175; P=0.05 t=1.95996

= BA1IAA1 = BA(0.0 mg l^{-1});IAA(0.0 mg l^{-1})
= BA1IAA2 = BA(0.0 mg l^{-1});IAA(0.01 mg l^{-1})
= BA1IAA3 = BA(0.0 mg l^{-1});IAA(0.1 mg l^{-1})
= BA1IAA4 = BA(0.0 mg l^{-1});IAA(1.0 mg l^{-1})
= BA2IAA1 = BA(0.01 mg l^{-1});IAA(0.0 mg l^{-1})
= BA2IAA2 = BA(0.01 mg l^{-1});IAA(0.01 mg l^{-1})
= BA2IAA3 = BA(0.01 mg l^{-1});IAA(0.1 mg l^{-1})
= BA2IAA4 = BA(0.01 mg l^{-1});IAA(1.0 mg l^{-1})
= BA3IAA1 = BA(0.1 mg l^{-1});IAA(0.0 mg l^{-1})
= BA3IAA2 = BA(0.1 mg l^{-1});IAA(0.01 mg l^{-1})
= BA3IAA3 = BA(0.1 mg l^{-1});IAA(0.1 mg l^{-1})
= BA3IAA4 = BA(0.1 mg l^{-1});IAA(1.0 mg l^{-1})
= BA4IAA1 = BA(1.0 mg l^{-1});IAA(0.0 mg l^{-1})
= BA4IAA2 = BA(1.0 mg l^{-1});IAA(0.01 mg l^{-1})
= BA4IAA3 = BA(1.0 mg l^{-1});IAA(0.1 mg l^{-1})
= BA4IAA4 = BA(1.0 mg l^{-1});IAA(1.0 mg l^{-1})

Experiment 2: The induction of direct embryogenesis in tetraploid potato cultivars

Of the material used in this experiment, the cultivars BP_1 and Buffelspoort showed no response and data was analyzed only for Atzimba. The interaction between the two main effects **medium** and **the initial time of incubation on standard initiation medium** (Table 19[a]) were analyzed. This interaction was significant in terms of embryoid frequency (Table 30b). The interaction showed that control treatment 2 induced a significantly higher embryoid yield (13.60012) compared to all other treatments. The LS/21 day treatment (2.5) also yielded a significantly higher embryoid frequency compared to LS/7 days (0.00046), LS/14 days (0.00046), LS/28 days (0.00046), [b]/14 days (0.00046), [b]/21 days (0.00046) [b]/28 days (0.00046) and control 1 (0.00046). Control 2 differed significantly from LS/21 days (4,047996).

The interaction was also significant in terms of anther response (Table 31b). The three treatments LS/7 days (5.7171), LS/21 days (4.1667) and control 2 (8.0035) all resulted in a significantly higher anther response compared to treatments [b]/7 days (0.8696), [b]/14 days (0.0012), [b]/21 days (0.0012), [b]/28 days (0.8), LS/28 days (0.0012) and control 1 (0.8333). Furthermore, control 2 yielded a significantly higher anther response compared to treatment LS/14 days (1.9048). No significance was detected between treatments LS/7 days, LS/21 days and control 2.

Table 30a Summary of analysis from regression analysis for variable embryoid frequency

Source	DF	Sum of squares	Mean square	F value
Regression	9	1391	154.59	13.28
Residual	225	2619	11.64	
Corrected Total	234	4011	17.14	

Table 30b Predictions from regression model for medium/incubation time interaction for variable embryoid frequency

Treatment	Embryoid frequency LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i)=LSMEAN(j) i/j	1	2	3	4	5	6	7	8	9	10
LS/7 days	0.00046	0.00964	1										
[b]/7 days	0.86957	0.66343	2	1.309887									
LS/14 days	0.00046	0.00964	3	0	1.309887								
[b]/14 days	0.00046	0.00921	4	0	1.309899	0							
LS/21 days	2.5	1.10122	5	2.269705	1.268203	2.269705	2.269712						
[b]/21 days	0.00046	0.00884	6	0	1.309909	0	0	2.269719					
LS/28 days	0.00046	0.00902	7	0	1.309904	0	0	2.269716	0				
[b]/28 days	0.00046	0.00884	8	0	1.309909	0	0	2.269719	0	0			
CONTROL 1	0.00046	0.00902	9	0	1.309904	0	0	2.269716	0	0	0		
CONTROL 2	13.60012	2.51129	10	5.415368	4.901183	5.415368	5.415372	4.047996	5.415374	5.415373	5.415374	5.415373	

P=0.01 t=2.6175; P=0.05 t=1.95996

Control 1 = initiation medium [a] / anthers transferred monthly to fresh medium [a]
Control 2 = initiation medium [a] / without monthly transfer to fresh medium [a]
LS = basal medium of Linsmaier and Skoog with same composition as initiation medium [a], but without any growth-regulators / anthers transferred monthly to fresh medium
[b] = embryoid regeneration medium with GA_3 / anthers transferred monthly to fresh medium
LS/x days = anthers were transferred after "x" days cultured on initiation medium [a] to LS
[b]/x days = anthers were transferred after "x" days on initiation medium [a] to regeneration medium [b]
"x" = indicated respectively 7, 14, 21 and 28 days

Table 31a Summary of analysis from regression analysis for variable anther response

Source	DF	Sum of squares	Mean square	F value
Regression	9	739	82.12	6.41
Residual	225	2882	12.81	
Corrected Total	234	3621	15.48	

Table 31b Predictions from regression model for medium/incubation time interaction for variable anther response

Treatment	Anther Response LSMEAN	Standard error LSMEAN	Pr > [T] H0:LSMEAN(i) =LSMEAN(j) i/j	1	2	3	4	5	6	7	8	9	10
LS/7 days	5.7171	1.8388	1										
[b]/7 days	0.8696	0.6959	2	2.465569									
LS/14 days	1.9048	1.0760	3	1.789406	0.807850								
[b]/14 days	0.0012	0.0159	4	3.108378	1.247555	1.768952							
LS/21 days	4.1667	1.4913	5	0.654862	2.003492	1.229993	2.793042						
[b]/21 days	0.0012	0.0153	6	3.108387	1.247579	1.768966	0	2.793054					
LS/28 days	0.0012	0.0156	7	3.108383	1.247567	1.768959	0	2.793048	0				
[b]/28 days	0.8000	0.6402	8	2.525398	0.073605	0.882392	1.247350	2.074486	0.316301	1.247365			
CONTROL 1	0.8333	0.6669	9	2.496829	0.037661	0.846426	1.247359	2.040493	1.247385	1.247372	0.036021		
CONTROL 2	8.0035	1.9957	10	0.842549	3.375316	2.689866	4.009644	1.540052	4.009653	4.009649	3.436996	3.407598	

P=0.01 t=2.6175; P=0.05 t=1.95996

Control 1 = initiation medium [a] / anthers transferred monthly to fresh medium [a]
Control 2 = initiation medium [a] / without monthly transfer to fresh medium [a]
LS = basal medium of Linsmaier and Skoog with same composition as initiation medium [a], but without any growth-regulators / anthers transferred monthly to fresh medium
[b] = embryoid regeneration medium with GA_3 / anthers transferred monthly to fresh medium
LS/x days = anthers were transferred after "x" days cultured on initiation medium [a] to LS
[b]/x days = anthers were transferred after "x" days on initiation medium [a] to regeneration medium [b]
"x" = indicated respectively 7, 14, 21 and 28 days

Table 32 Summary of analysis from regression analysis for variable anther productivity

Source	DF	Sum of squares	Mean square	F value
Regression	6	541.7	90.3	0.51
Residual	4	711.8	177.9	
Corrected Total	10	1253.4	125.3	

IV DISCUSSION

Experiment 1: The effect of differential auxin and cytokinin treatments on the androgenetic response of various potato genotypes

TRIAL 1

Results from trial 1 indicate that BA had to be present in the initiation medium of anther cultures of dihaploid breeding line 87.2002/3 for increased embryoid production. A BA concentration of 1.5 mg l^{-1} proved best. Neither the absence or presence of IAA seemed to affect anther response (Tables 25b and 25c). This correlates with the results of MAHESHWARI, TYAGI and MALHORTA (1980) and WENZEL and FOROUGHI-WEHR (1984), that auxin addition to the medium of *Solanum tuberosum* anther cultures is not essential, while cytokinin addition is critical. It can also be concluded from Table 25d that the auxin IAA had no effect on *in vitro* embryogenesis in either the absence of BA or when relatively low levels of BA (0.5 mg l^{-1} and 1.0 mg l^{-1}) were present. IAA only increased the androgenetic response of this breeding line when BA was present at a relatively high concentration of 1.5 mg l^{-1}. The latter was concluded from the fact that the interaction between the combination 1.5 mg l^{-1}BA/0.1 mg l^{-1}IAA differed significantly from 1.5 mg l^{-1}BA/0.1 mg l^{-1}IAA (Table 24d). From Table 26d it was indicated that a BA/IAA combination of 0.0 mg l^{-1}BA/1.0 mg l^{-1}IAA (BA1IAA3) yielded a significantly higher anther productivity compared to both 0.0 mg l^{-1}BA/0.0 mg l^{-1}IAA (BA1IAA1) and 0.0 mg l^{-1}BA/1.5 mg l^{-1}IAA (BA1IAA4). This suggests that, once embryoid production is initiated, an IAA concentration of 1.0 mg l^{-1} could lead to an increase in embryoid production and anther response. Thus, an IAA concentration of 1.0 mg l^{-1} proved to be optimal. The optimal BA/IAA combination for maximal embryoid production was 1.5 mg l^{-1}BA/1.0 mg l^{-1}IAA. This differed from both the composition of the standard initiation medium used in local anther culture experiments and the optimal growth regulator concentrations

reported by TIANEN (1993) (1.0 mg l^{-1} BA/0.1 mg l^{-1}IAA). Most researchers seldom included BA at levels lower than 1.0 mg l^{-1}, going as high as 2.5 mg l^{-1} (WENZEL and FOROUGHI-WEHR, 1984; POWELL and UHRIG, 1987; TIAINEN, 1993). It could be that the dihaploid breeding line 87.2002/3 required slightly higher BA and IAA concentrations, due to specific genotype requirements.

TRIAL 2

Results from Trial 2 indicate that in the absence of BA in the initiation medium, the auxin IAA could significantly increase both the embryoid frequency (Table 27d) and the anther response (Table 28d) of the dihaploid breeding line 87.2002/3. No specific IAA concentration could be defined from these results, especially at relatively low BA concentrations (0.01 mg l^{-1} and 0.1 mg l^{-1}). However, at a relatively high BA concentration (1.0 mg l^{-1}), a concentration of 0.1 mg l^{-1} IAA increased both embryoid frequency (Table 27d) and anther response (Table 28d). Furthermore, from Table 28d it can be concluded that either none or relatively low IAA concentrations (0.1 mg l^{-1}), combined with a relatively high BA concentration of 1.0 mg l^{-1}, increased anther response. The latter also indicates the importance of BA addition for increased anther response. Thus, according to this trial, IAA addition to the initiation medium at 0.1 mg l^{-1} proved essential for increased androgenetic response. This finding differed from that indicated in Trial 1, namely that BA addition was essential while IAA seemed to have no effect on anther response.

Due to the fact that the interaction between the two variables "medium" and "incubation time" was not significant in terms of anther productivity, it was concluded that once embryoid production was initiated, the addition of a specific IAA concentration to a specific BA concentration would not increase the androgenetic response of this breeding line.

From **both trials** of experiment 1 it was concluded that the addition of the cytokinin BA was essential for increased androgenetic response *in vitro*. The optimal BA concentration varied, however, between the two trials. This variation and difference in response could possibly be due to specific genotype requirements, or to various external factors - either single or in combination - such as the occurrence of varying culture conditions during the performance of the two trials; varying growth- and glass house conditions; flowers probably were selected at different periods of either (i) their microspore developmental stage ór (ii) at different times during the flowering period of the donor plants; or their hormonal requirements may have been different.

In both trials IAA proved to have an increasing effect on anther response at some stage, either (i) in combination with a relatively high BA concentration (1.5 mg l^{-1}) (Trial 1 with an optimal IAA concentration of 1.0 mg l^{-1}), or (ii) IAA concentrations of either 0.1 mg l^{-1} or 1.0 mg l^{-1} used in the absence of BA (Trial 2), or (iii) 0.1 mg l^{-1} IAA combined with the maximum BA concentration 1.0 mg l^{-1} (Trial 2). Thus, in both Trial 1 and Trial 2 the increasing effect of IAA on anther response was observed when the respective IAA concentrations was combined with the maximum BA concentrations utilized in the two trials. The findings concerning the importance of IAA addition to the initiation medium, agrees with that of HEYNOLDS (1986) who reported the necessity of the presence of IAA in the nutrient medium for embryogenesis. According to him, increasing IAA concentrations enhanced embryoid induction. He observed a maximal embryoid yield when an IAA concentration as high as 10 mg l^{-1} was utilized in anther cultures of *Solanum carolinense*.

In terms of embryoid frequency, the results in Table 24d (Trial 1) and Table 27d (Trial 2) indicated that the highest embryoid frequency (or embryoid yield) was obtained at the highest BA concentration used in the respective trials (Trial 1 = 1.5 mg l^{-1}BA and Trial 2 = 1.0 mg l^{-1}BA).

The corresponding IAA concentrations combined with these BA concentrations differed. From these results it can be concluded that the highest embryoid yields are obtained at a relatively high BA concentration in combination with varying IAA concentrations. This again underlined the major effect (although not exclusive) of BA to that of IAA in the initiation medium for improved androgenetic response *in vitro*. It must be kept in mind, however, that in this study the effect of IAA was only analyzed in concentrations as high as 1.5 mg l^{-1}, while REYNOLDS (1986) indicated an optimal concentration of 10 mg l^{-1}.

Experiment 2: Direct Embryoids in Tetraploids

In terms of embryoid frequency the LS/21 day treatment gave a significantly higher embryoid yield compared to all other treatments, **with the exception of control 2**. The latter yielded the highest embryoid frequency. This implies that the highest embryoid production is obtained when anthers from the tetraploid cultivar Atzimba are cultured on the initiation medium (Table 19[a]) for the three month duration of this experiment, without either (i) anther transfer to fresh initiation medium, or (ii) at different time intervals to the regeneration medium (Table 19[b]) or LS-medium (the latter contained no growth regulators). The initiation medium (Table 19[a]) utilized in control 2, contained the growth regulators IAA, BA, glutamine and asparagine (as indicated in Table 19[a]). These growth regulators, especially IAA and BA, proved very unstable and break down and/or are metabolized after a short time in culture (HEYLEN, VENDRIG and VAN ONCKELEN, 1991). This implies that either the addition of the exogenous growth regulators have a determining and increasing role in microspore derived embryoid production early in the culture period - that is for the first seven to 21 days of the culture period, or that anthers should be transferred to fresh medium with new growth regulators much earlier during the culture period.

In terms of anther response (Table 31b) approximately the same response was observed from treatments LS/7 days, LS/21 days and control treatment 2. These three treatments did not differ significantly from each other. Control 2 also yielded a significantly higher anther response compared to [b]/7 days, [b]/14 days, [b]/21 days, [b]/28 days and LS/28 days. The initial culture of Atzimba anthers for the first 7 to 21 days on the initiation medium (Table 19[a]) induced more anthers for androgenetic response. After 28 days of culture, the growth regulators would probably already have been used or broken down. This initiating effect of the growth regulators during the first 7 - 21 days of the culture period was confirmed and emphasized by the microtome sections from cultured anthers of breeding line 87.2002/3 as discussed is Chapter 2. From these slides embryoid development was detected as early as 7 days of anther culture, with the most prominent embryoid development after 14 days of culture.

The transfer of anthers from initiation medium [a] to the regeneration medium [b] containing GA_3, never increased the frequency of embryoid production or anther response in anther cultures of the responsive tetraploid cultivar Atzimba. This agrees with the results by TIANEN (1993) and NITSCH (1969), namely that GA_3 does not promote embryogenesis of potato and tobacco anther cultures.

V CONCLUSIONS

The exogenous applied growth regulators BA, IAA, glutamine and asparagine, as used in these experiments, play a determining and enhancing role in microspore derived embryoid production and overall androgenetic response, especially during the first 7 to 21 days of the culture period of responsive potato genotypes. The inclusion of BA and IAA are essential, but optimal concentrations vary due to specific genotype requirements and/or due to one or more external factors. BA concentrations between 0.1 mg l^{-1} and 1.5 mg l^{-1} and IAA concentrations ranging from 0.1 mg l^{-1} to 1.0 mg l^{-1} proved important. When considering its inclusion in initiation medium, the specific combinations of different concentrations as well as its interaction should be considered.

As growth regulators such as BA and IAA are metabolized in culture medium as well as the fact that a significantly higher embryoid frequency and anther response were obtained from anthers cultured for 21 days on the initiation medium, it is important that responsive anthers should be transferred to fresh medium at shorter intervals rather than on a four weekly basis. As androgenetic responses seemed most pronounced during the first 7 to 21 days, anthers could be transferred, for instance, on a two-weekly basis. The fresh, metabolically active growth regulators would, however, only increase embryogenesis while microspore derived embryoid production could still be induced. Due to the fact that the stage of microspore development, and therefore microsporogenesis, is not synchronized in any one anther, the induction of microspore derived embryoid production would not be "completed" simultaneously in anthers of amenable genotypes. Therefore, the transfer of anthers to fresh medium containing active growth regulators is essential to provide those microspores (in which induction of embryogenesis is not yet completed) with the necessary nutrients. The gibberellin GA_3 had no enhancing

effect on either embryoid production or anther response in anther cultures of tetraploid potato genotypes.

VI RECOMMENDATIONS FOR FUTURE STUDIES

In Experiment 1 the same optimal cold pre-treatments were performed on all genotypes, various combinations of a wide range of concentrations of the growth regulators BA and IAA were tested and two different growth seasons were utilized (which excludes the possibility that the flowers from the first flowering season could perform for instance better under *in vitro* conditions compared to those from the flowering season much later in the year). Despite these conditions, the tetraploid cultivar Atzimba and diploid wild species *S. canasense* still showed no response. The various optimal BA/IAA combinations - resulting from this data - should be used in liquid cultures due to Atzimba's previous success with liquid cultures (see Chapter 3). Together with this, care should be taken to select flowers correlating with the uninucleate stage of microspore development (as discussed in Chapter 2). Therefore, a pre-determination should first be carried out on all genotypes for a flower bud length most representative of the uninucleate stage of microspore development (Chapter 2). The effect of higher IAA concentrations in anther cultures of wild species should receive more attention. Care should, however, be taken concerning the latter as concentrations higher than 0.1 mg l^{-1} of certain auxins such as 2,4-D favours callus regeneration rather than direct embryogenesis (DUNWELL, 1985).

REFERENCES

AFELE, J.C., KANNENBERG, L.W., KEATS, R., SOHOTA, S. and SWANSON, E.B. 01992. Increased induction of microspore embryos following manipulation of donor plant environment and culture temperature in corn (*Zea mays* L.). *Plant Cell, Tissue and Organ Culture* **28**: 87 - 90.

ANAGNOSTAKIS, S.L. 1974. Haploid plants from anthers of tobacco-enhancement with charcoal. *Planta* **115**: 281 - 283.

BAJAJ, Y.P.S. and SOPORY, S.K. 1986. Biotechnology of Potato Improvement. *In* BAJAJ, Y.P.S., Biotechnology in Agriculture and Forestry. Crops I: 429 - 454. Springer Verlag, Berlin. ISBN 3-540-15842-1.

BATTY, N.J.P. and DUNWELL, H.M. 1986. The effects of various pre-treatments on the response of *Solanum tuberosum* cv. H3703 to anther culture. *In* EAPR/Eucarpia Breeding and Variety Assessment meeting, Cambridge.

BATTY, N.J.P. and DUNWELL, H.M. 1989. Effect of maltose on the response of potato anthers in culture. *Plant Cell, Tissue and Organ Culture* **18 (2)**: 221 - 226.

BENNET, M.D. and HUGHES, W.G. 1972. Additional mitosis in wheat pollen induced by ethrel. *Nature* **240**: 566 - 568.

BIDDINGTON, N.L. and ROBINSON, Helen T. 1990. Variations in response to high temperature treatments in anther culture of Brussels sprouts. *Plant Cell, Tissue and Organ Culture* **22**: 48 - 54.

BINDING, H. 1972. Nuclear and cell divisions in isolated pollen of *Petunia hybrida* in agar suspension cultures. *Nature New Biology* **237**: 283 - 285.

BONIERBALE, M.W., PLAISTED, R.L. and TANKSLEY, S.D. 1988. RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. *Genetics Society of America* **120**: 1095 - 1103.

BRINK, J.A., DE BRUYN, M.H., VAN RHYN, C.C. and FERREIRA, D.I. 1989. Regeneration of callus and embryoids from potato anthers. *Proceedings of Potato Research Symposium*, 33-37.

BROWN, C., BROOKS, F.V., PEARSON, D. and MATHIAS, R.J. 1989. Control of embryogenesis and organogenesis in immature wheat embryo callus using increased medium osmolarity and abscisic acid. *Journal of Plant Physiology* **133**: 727 - 733.

CAI, Q., SZAREJKO, I., POLOK, K. and MALUSZYNSKI, M. 1992. The effect of sugars and growth regulators on embryoid and plant regeneration from barley anther culture. *Plant Breeding* **3**: 218 - 226.

CALIGARI, P.D.S., POWELL, W., LIDDELL, K., DE MAINE, M.J. and SWAN, G.E.L. 1988. Methods and strategies for detecting *Solanum tuberosum* dihaploids in interspecific crosses with *S. phureja*. *Annals of Applied Biology* **112**: 323 -328.

CALLEBERG, Eva K. and JOHANSSON, L.B. 1993. The effect of starch and incubation temperature in anther culture of potato. *Plant Cell, Tissue and Organ Culture* **32**: 27 - 34.

CAPPADOCIA, M., CHENG, D.S.K. and LUDLEM-SIMONETTE, R. 1984. Plant regeneration from in vitro culture of anthers of *Solanum chacoense* Bitrt. and interspecific diploid hybrids *S. tuberosum* L. x *S. chacoense* Bitt. *Theoretical and Applied Genetics* **69**: 131 - 143.

CHASE, S.S. 1963. Analytic breeding in *Solanum tuberosum* L. A scheme utilizing parthenotes and other diploid stocks. *Canadian Journal of Genetics and Cytology* **5**: 359 - 363.

CHEN, Y., WANG, R.F., TIAN, W.Z., ZUO, Q.X., ZENG, S.W., LU, D.Y., and ZHANG, G.H. 1980. Studies on pollen culture *in vitro* and induction of plantlets of *Oryza sativa* L. *In* DAVIES, D.R. and HOPWOOD, D.A. (eds.), The plant genome : proceedings of the fourth John Innes Symposium and second international Haploid Conference, 245. John Innes Institute, Norwich. ISBN 0-950-68440-6.

CLAPHAM, D. 1973. Haploid *Hordeum* plants from anthers in vitro. *Zeitschrift für Pflanzenzuchtung* **69**: 142 - 155.

CORDUAN, G. 1975. Regeneration of anther derived plants of *Hyoscyamus niger* L. *Planta* **127**: 27 - 36.

DODDS, K.S. 1962. Classification of cultivated potatoes. *In* CORRELL, D.S. (ed.), The potato and its wild relatives : section Tuberarium of the genus Solanum. Texas Research Foundation **4**: 514 - 539. LC 68000538.

DODDS, J.H. and ROBERTS, L.W. 1985. Anther and pollen cultures. *In* DODDS, J.H. and ROBERTS, L.W.(eds), Experiments in Plant Tissue Culture, Second edition, Cambridge, 157 - 171. ISBN 0-521-30478-4.

DUNCAN, E.J. and HEBERLE, E. 1976. Effect of temperature shock on nuclear phenomena in microspores of *Nicotiana tabacum* and consequently on plantlet production. *Protoplasma* **90**: 173 - 177.

DUNWELL, J.M. 1976. A comparative study of environmental and developmental factors which influence embryo induction and growth in cultured anthers of *Nicotiana tabacum*. *Environmental Experimental Botany*. **16**: 109 - 118.

DUNWELL, J.M. 1979. Anther culture in *Nicotiana tabacum*: The role of the culture vessel atmosphere in pollen embryo induction and growth. *Journal of Experimental Botany* **30**: 419 - 428.

DUNWELL, J.M. 1985. Embryogenesis from pollen *in vitro*. *In* ZAITLIN, M., DAY, P. and HOLLAENDER, A. (eds.), Biotechnology in Plant Science, 49 - 76. Academic Press, Incorporated. ISBN 0-127-75310-9.

DUNWELL, J.M. and SUNDERLAND, N. 1973. Anther culture of Solanum tuberosum L. *Euphytica* **22**: 317 - 323.

FAHN, A. 1974. Reproductive Organs (The Flower). *In* Plant Anatomy, Second edition, 425 - 506. Pergamon Press, New York.

FEDER, N. and O'BRIEN, T.P. 1968. Plant microtechnique: some principles and new methods. *American Journal of Botany* **55 (1)**: 123 - 142.

FERREIRA, D.I. and ZELCER, A. 1988. Advances in protoplast research on *Solanum*. *International Review of Cytology* **115**: 1 - 60.

FOROUGHI-WEHR, B. and WENZEL, G. 1993. Andro- and Parthenogenesis. *In* HAYWARD, M.D., BOSEMARK, N.O. and ROMAYOSA, I. (eds), Plant Breeding: Principles and Prospects **17**: 261 - 277. London: Chapman and Hall. ISBN 0-412-43390-7.

FOROUGHI-WEHR, B., WILSON, H.M., MIX, G. and GAUL, H. 1977. Monohaploid plants from anthers of dihaploid genotype of *Solanum tuberosum* L. *Euphytica* **26**: 361 - 368.

GILL, B.S., W. KAM-MORGAN, L.N. and SHEPARD, J.F. 1986. Origin of chromosomal and phenotypic variation in potato protoclones. *Journal of Heredity* **77**: 13 - 16.

GRESSHOFF, P.M. and DOY, C.H. 1972a. Haploid *Arabidopsis thaliana* callus and plants from anther culture. *Australian Journal of Biological Science* **25**: 259 - 264.

GRESSHOFF, P.M. and DOY, C.H. 1972b. Development and differentiation of haploid *Lycopersicon esculentum*. *Planta* **107**: 161 - 170.

GRESSHOFF, P.M. and DOY, C.H. 1974. Derivation of a haploid cell line from *Vitis vinifera* and the importance of the stage of meiotic development of anthers for haploid culture of this and other genera. *Zeitschrift für Pflanzenphysiologie* **73**: 132 - 144.

GUHA, S. and MAHESHWARI, S.C. 1964. *In vitro* production of embryos from anthers of *Datura*. *Nature* **204**: 497.

GUHA, S. and MAHESHWARI, S.C. 1966. Cell division and differentiation of embryos in the pollen grains of *Datura* in vitro. *Nature* **212**: 97 - 98.

GUHA, S. and MAHESHWARI, S.C. 1967. Development of embryoids from pollen grains of *Datura* in vitro. *Phytomorphology* **17**: 454 - 461.

HAUNG, B. and SUNDERLAND, N. 1982. Temperature-stress pre-treatment in barley anther culture. *Annals of Botany* **49**: 77 - 78.

HAWKES, J.G. 1978. History of the potato. *In* HARRIS, P.M. (ed.), The Potato Crop: the scientific basis for improvement, 1 - 14. London: Chapman and Hall. ISBN 0-412-12830-6.

HAWKES, J.G. 1979. Genetic poverty of the potato in Europe. Proc. Conf. Broad. Gen. Base Crops, 19 - 27. Wageningen, 1978.

HAWKES, J.G. and FRANCISCO-ORTEGA, J. 1993. The early history of the potato in Europe. *Euphytica* **70**: 1 - 7.

HEBERLE-BORS, E. 1982. On the time of embryogenic pollen grain induction during sexual development in *Nicotiana tabacum* L. plants. *Planta* **156**: 402 - 406.

HEBERLE, E. and REINERT, J. 1977. Factors of haploid production by isolated pollen cultures. *Naturwissenschaften* **64**: 100.

HERMSEN, J.G.T. and VERDENIUS, J. 1973. Selection from *Solanum tuberosum* group Phureja of genotypes combining high frequency haploid induction with homosygosity for embryo-spot. *Euphytica* **22**: 244 - 259.

HEYLEN, Christine, VENDRIG, J.C. and VAN ONCKELEN, H. 1991. The accumulation and metabolism of plant growth regulators during organogenesis in cultures of thin cell layers of *Nicotiana tabacum*. *Physiologia Plantarum* **83**: 578 - 584.

HORNER, M., McCOMB, J.A., McCOMB, A.J. and STREET, H.E. 1977. Ethylene production and plantlet formation by *Nicotiana* anthers cultured in the presence and absence of charcoal. *Journal of Experimental Botany* **28**: 1365 - 1372.

HORNER, M. and PRATT, M.L. 1979. Amino acid analysis of in vivo and androgenic anthers of *Nicotiana tabacum*. *Protoplasma* **98**: 279 - 282.

HOUGAS, R.W. and PELOQUIN, S.J. 1958. The potential of potato haploids in breeding and genetic research. *American Potato Journal* **35**: 701 - 707.

HOUGAS, R.W., PELOQUIN, S.J. and GABERT, A.C. 1964. Effect of seed-parent and pollinator on frequency of haploids in *Solanum tuberosum*. *Crop Science* **4**: 593 - 595.

HOUGAS, R.W., PELOQUIN, S.J. and ROSS, R.W. 1958. Haploids of the common potato. *Journal of Heredity* **49**: 103 - 106.

HOWARD, H.W. 1973. Calyx forms in dihaploids in relation to the origin of *Solanum tuberosum*. *Potato Research* **16**: 43 - 46.

HOWARD, H.W. 1978. The production of new varieties. *In* HARRIS, P.M. (ed.), The Potato Crop: the scientific basis for improvement, 607 - 646. London: Chapman and Hall. ISBN 0-412-12830-6

HUGHES, K.W., BELL, S.L. and CAPONETTI, J.D. 1975. Anther-derived haploids of the African violet. *Canadian Journal of Botany* **53**: 1442 - 1444.

HUNTER, C.P. 1985. The effect of anther orientation on the production of microspore-derived embryoids and plants of *Hordeum vulgare* cv. Sabarlis. *Plant Cell Reports* **4**: 267 - 268.

IRIKURA, Y. 1975. Induction of haploid plants by anther culture in tuber-bearing species and interspecific hybrids of *Solanum*. *Potato Research* **18**: 133 -140.

JACOBSEN, E. and SOPORY, S.K. 1978. The influence and possible recombination of genotypes on the production of microspore embryoids in anther cultures of *Solanum tuberosum* and dihaploid hybrids. *Theoretical and Applied Genetics* **52**: 119 - 123.

JOHANSSON, L. 1983. Effects of activated charcoal in anther cultures. *Physiologia Plantarum* **59**: 397 - 403.

JOHANSSON, L. 1986. Improved methods for induction of embryogenesis in anther cultures of *Solanum tuberosum*. *Potato Research* **29**: 179 - 190.

JOHANSSON, L.B. 1987. Increased induction of embryogenesis and regeneration in anther cultures of *Solanum tuberosum* L. *Potato Research* **31**: 145 - 149.

JOHANSSON, L.B. 1988. Increased induction of embryogenesis and regeneration in anther cultures of *Solanum tuberosum* L. *Potato Research* **31**: 145 - 149.

JOHANSSON, L. and ERIKSSON, T. 1977. Induced embryo formation in anther cultures of several *Anemone* species. *Physiologia Plantarum* **40**: 172 - 174.

JOHRI, B.M. 1984. Embryology of angiosperms. Springer-Verlag, 55. New York. ISBN 0-387-12739-9.

KASPERBAUER, M.J. and COLLINS, G.B. 1974. Anther-derived haploids in tobacco: evaluation of procedures. *Crop Science* **14**: 305 - 307.

KELLER, W.A. and ARMSTRONG, K.C. 1977. Embryogenesis and plant regeneration in *Brassica napus* anther cultures. *Canadian Journal of Botany* **55**: 1383 - 1388.

KELLER, W.A. and ARMSTRONG, K.C. 1978. High frequency production of microspore-derived plants from *Brassica napus* anther cultures. *Zeitschrift für Pflanzenzuchtung* **80**: 100 - 108.

KELLER, W.A. and ARMSTRONG, K.C. 1979. Stimulation of embryogenesis and haploid production in *Brassica campestris* anther cultures by elevated temperature treatments. *Theoretical and Applied Genetics* **55**: 65 - 67.

KELLER, W.A., RAJHATHY, T. and LACAPRA, J. 1975. In vitro production of plants from pollen in *Brassica campestris*. *Canadian Journal of Genetic Cytology* **17**: 655 - 666.

KELLER, W.A. and STRINGAM, G.R. 1978. Production and utilization of microspore-derived haploid plants. *In* THORPE, T.A. (ed.), Frontiers of Plant Tissue Culture 1978: proceedings of the 4th International Congress of Plant Tissue and Cell Culture, 113 - 122. Calgary, Canada.

KIMBER, G. and RILEY, R. 1963. Haploid angiosperms. *Botanical Reviews*. **29**: 480 - 531.

KIRCH, H.H., UHRIG, H., LOTTSPEICH, F., SALAMINI, F. and THOMPSON, R.D. 1989. Characterization of proteins associated with self-incompatibility in *Solanum tuberosum*. *Theoretical and Applied Genetics* **78**: 581 -588.

KOHLENBACH, H.W. and WERNICKE, W. 1978. Investigations on the inhibitory effect of agar and the function of active carbon in anther culture. *Zeitschrift für Pflanzenphysiologie* **86**: 463 - 472.

KOHLER, F. and WENZEL, G. 1985. Regeneration of isolated barley microspores in conditioned media and trials to characterize the responsible factor. *Journal of Plant Physiology* **121**: 181 - 191.

LAZAR, M.D., SCHAEFFER, G.W. and BAENZIGER, P.S. 1984. Cultivar and cultivar x environment effects on the development of callus and polyhaploid plants from anther cultures of wheat. *Theoretical and Applied Genetics* **67**: 273 - 277.

LAZAR, M.D., SCHAEFFER, G.W. and BAENZIGER, P.S. 1985. The physical environment in relation to high frequency callus and plantlet development in anther cultures of wheat (Triticum aestivum L.) cv. Chris. *Journal of Plant Physiology* **121**: 103 - 109.

LINSMAIER, E.M. and SKOOG, F. 1965. Organic growth factor requirements of tobacco tissue cultures. *Physiologia Plantarum* **18**: 100 - 127.

LIU, C. and DOUCHES, D.S. 1993. Production of haploids of potato (*Solanum tuberosum* subsp. *tuberosum*) and their identification with electrophoretic analysis. *Euphytica* **70**: 113 - 126.

MAHESHWARI, S.C., RASHID, A. and TYAGI, A.K. 1982. Haploids from pollen grains-retrospect and prospect. *American Journal of Botany* **69**: 865 - 878.

MAHESHWARI, S.C., TYAGI, A.K. and MALHOTRA, K. 1980. Induction of Haploidy from Pollen Grains in Angiosperms - the Current Status. *Theoretical and Applied Genetics* **58**: 193 - 206.

MALHORTA, K. and MAHESHWARI, S.C. 1977. Enhancement by cold treatment of pollen embryoid development in *Petunia hybrida*. *Zeitschrift für Pflanzenphysiologie* **85**: 177 - 180.

MEYER, R. SALAMINI, F. and UHRIG, H. 1993. Isolation and characterization of potato clones generating a high frequency of monohaploid or homozygous diploid androgenetic plants. *Theoretical and Applied Genetics* **85**: 905 - 912.

MILLER, S.A. and LIPSCHUTZ, L. 1984. Potato. *In* AMMIRATO, P.V., EVANS, D.A., SHARP, W.R. and YAMADA, Y. (eds.), Handbook of plant cell culture, **3**: 291 - 326. Maxwell McMillan International, New York. ISBN 0-07-001583-X.

MIX, G. 1983. Production of dihaploid plantlets from anthers of autotetraploid genotypes of *Solanum tuberosum*. *Potato Research* **26**: 63 - 67.

MORRISON, R.A. and EVANS, D.A. 1988. Haploid plants from tissue culture: New plant varieties in a shortened time frame. *Biotechnology* **6**: 684 - 689.

MORRISON, R.A., KONING, R.E. and EVANS, D.A. 1986. Anther culture of an interspecific hybrid of *Capsicum*. *Journal of Plant Physiology* **126**: 1 - 9.

MURASHIGE, T. and SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**: 473 - 497.

NAKAMURA, A. and ITAGAKI, R. 1973. Anther culture in *Nicotiana* and the characteristics of the haploid plants. *Japanese Journal of Breeding* **23**: 71 - 78.

NIIZEKI, M. and GRANT, W.F. 1971. Callus, plantlet formation, and polyploidy from cultured anthers of *Lotus* and *Nicotiana*. *Canadian Journal of Botany* **49**: 2041 - 2051.

NIIZEKI, M. and OONO, K. 1971. Rice plants obtained by anther culture. Colloq. Int. CNRS (Paris) **193**: 251 - 257.

NITSCH, C. 1974. Pollen culture - a new technique for mass production of haploid and homozygous plants. *In* KASHA, K.J. (ed.), Haploids in Higher Plants: advances and potential : preceedings of the First International Symposium, 123 - 135. Guelph, Ontario, Canada.

NITSCH, C. 1981. Production of isogenic lines: basic technical aspects of androgenesis. *Plant Tissue and Cell Culture*, 241-249.

NITSCH, J.P. 1951. Growth and development *in vitro* of excised ovaries. *American Journal of Botany* **38**: 566 - 577.

NITSCH, J.P. 1969. Experimental androgenesis in *Nicotiana*. *Phytomorphology* **19**: 390 - 404.

NITSCH, J.P. and NITSCH, C. 1969. Haploid plants from pollen grains. *Science* **163**: 85 - 87.

PELLETIER, G. and HENRY, Y. 1974. Cold pretreating on flower buds of *Nicotiana tabacum* L. *Haploid Information Service* **10**: 5 - 8.

PIERIK, R.L.M. 1993. The production of haploids. *In* PIERIK, R.L.M. (ed.), In vitro culture of higher plants, 243 - 257. Martinus Nijhoff, Wageningen. ISBN 90-247-3530-0.

POWELL, W., BORRINO, E.M. and GOODALL, V. 1988. The effect of anther orientation on microspore-derived plant production in barley (*Hordeum vulgare* L.). *Euphytica* **38**: 159 - 163.

POWELL, W. and UHRIG, H. 1987. Anther culture of *Solanum* genotypes. *Plant Cell, Tissue and Organ Culture* **11**: 13 - 24.

RAGHAVEN, V. 1975. Induction of haploid plants from anther cultures of henbane. *Zeitschrift für Pflanzenphysiologie* **76**: 89 - 92.

RAGHAVEN, V. 1976. Role of the generative cell in androgenesis in henbane. *Science* **191**: 388 - 389.

RASCO Jr., E.T., PLAISTED, R.L. and EWING, E.E. 1980. Photoperiod response and earliness of *Solanum tuberosum* ssp. *andigena* after six cycles of recurrent selection for adaption to long days. *American Potato Journal* **57**: 435 - 447.

REINERT, J., BAJAJ, Y.P.S. and HEBERLE, E. 1975. Induction of haploid tobacco plants from isolated pollen. *Protoplasma* **84**: 191 - 196.

REYNOLDS, T.L. 1986. Pollen embryogenesis in anther cultures of *Solanum carolinense* L. *Plant Cell Reports*, **5**: 273 - 275.

RICKEMAN, V.S. and DESBOROUGH, Sharon L. 1978. Elucidation of the evolution and taxonomy of cultivated potatoes with electrophoresis. I. Groups *Tuberosum*, *Andigena*, *Phureja* and *Stenotomum*. *Theoretical and Applied Genetics* **52**: 217 - 220.

ROSS, H. 1986. Potato Breeding - Problems and Perspectives. *In* FREISING, W.H. and RöBBELEN, G. (eds), Advances in Plant Breeding, 1 - 132. Supplement 13 to Journal of Plant Breeding, 1986 Verlag Paul Parey, Berlin and Hamburg. ISBN 3-489-61110-1.

SANGWAN, R.S. and NORREEL, B. 1975. Induction of plants from pollen grains of *Petunia* cultured *in vitro*. *Nature* **257**: 222 - 224.

SANGWAN, R.S. and SANGWAN-NORREEL Brigitte, S. 1987. Biochemical Cytology of Pollen Embryogenesis. *International Review of Cytology* **107**: 221 - 272.

SANGWAN-NORREEL, Brigitte, S. 1977. Androgenic stimulating factors in the anther and isolated pollen grain culture of *Datura innoxia* Mill. *Journal of Experimental Botany* **28**: 843 - 852.

SHANNON, P.R.M., NICHOLSON, A.E., DUNWELL, J.M. and DAVIES, D.R. 1985. Effect of anther orientation on microspore callus production in barley (*Hordeum vulgare* L.). *Plant Cell, Tissue and Organ Culture* **4**: 271 - 280.

SHARP, W.R., RASKIN, R.S. and SOMMER, H.E. 1972. The use of nurse culture in the development of haploid clones in tomato. *Planta* **104**: 357 - 361.

SIMON, P.W. and PELOQUIN, S.J. 1977. The influence of paternal species on the origin of callus in anther culture of *Solanum* hybrids. *Theoretical and Applied Genetics* **50**: 53 - 56.

SKOOG, F. and MILLER, C.O. 1957. Chemical regulation of growth and organ formation in plant tissue cultured in vitro. *Symposium of the Society for Experimental Biology* **11**: 118 - 131.

SNIDER, Karen, T. and VEILLEUX, R.E. 1994. Factors affecting variability in anther culture and in conversion of androgenic embryos of *Solanum phureja*. *Plant Cell, Tissue and Organ Culture* **36**: 345 - 354.

SOPORY, S.K. 1977. Development of embrycids in isolated pollen culture of dihaploid *Solanum tuberosum*. *Zeitschrift für Pflanzenphysiologie* **84**: 453 - 457.

SOPORY, S.K. 1979. Effect of sucrose, hormones, and metabolic inhibitors on the development of pollen embryoids in anther culture of dihaploid *Solanum tuberosum*. *Canadian Journal of Botany* **57**: 2691 - 2694.

SOPORY, S.K. and BAJAJ, Y.P.S. 1987. Anther Culture and Haploid Production in Potato. *In* BAJAJ, Y.P.S. (ed.), Biotechnology in Agriculture and Forestry **3**: 89 - 104. Potato, Springer Verlag, Berlin, Heidelberg.

SOPORY, S.K., JACOBSEN, E. and WENZEL, G. 1978. Production of monohaploid embryoids and plantlets in cultured anthers of *Solanum tuberosum*. *Plant Science Letters* **12**: 47 -54.

SOPORY, S.K. and MAHESHWARI, S.C. 1976. Development of pollen embryoids in anther cultures of *Datura innoxia*. 1. General observations and effects of physical factors. *Journal of Experimental Botany* **27**: 49 - 57.

SOPORY, S.K. and ROGAN, P.G. 1976. Induction of pollen divisions and embryoid formation in anther cultures of some dihaploid clones of *Solanum tuberosum*. *Zeitschrift für Pflanzenphysiologie* **80**: 77 - 80.

STANSFIELD, W.D. 1983. The physical basis of heredity. *In* ZAYATZ, E. and GRICE, M. (eds.), Schaum's Outline of Theory and Problems of Genetics, Second edition, 1 - 18. McGraw-Hill, Incorporated. ISBN 0-070-60845-8.

SUNDERLAND, N. 1971. Anther culture: a progress report. *Scientific Progress* **59**: 527 - 549.

SUNDERLAND, N. 1974. Pollen and anther culture. *In* STREET, H.E. (ed.), Plant Tissue and Cell Culture, 205 - 240. University of California Press, 1973. ISBN 0-520-02411-7.

SUNDERLAND, N. and DUNWELL, J.M. 1977. Anther and pollen culture. *In* STREET, H.E. (ed.), Plant Tissue and Cell Culture, 223 - 265. University of California Press, 1973. ISBN 0-520-02411-7.

SUNDERLAND, N. and ROBERTS, M. 1977. New approach to pollen culture. *Nature* **270**: 236 - 238.

SUNDERLAND, N. and WILDON, D.C. 1979. A note on the pretreatment of excised flower buds in float culture of *Hyoscyamus* anthers. *Plant Science Letters* **15**: 169 -175.

TIAINEN, Tiana 1993. The influence of hormones on anther culture response of tetraploid potato (*Solanum tuberosum* L.). *Plant Science* **88**: 83 - 90.

TYAGI, A.K., RASHID, A. and MAHESHWARI, S.C. 1979. High frequency production of embryos in *Datura innoxia* from isolated pollen grains by combined cold treatment and serial culture of anthers in liquid medium. *Protoplasma* **99**: 11 - 17.

TYAGI, A.K., RASHID, A. and MAHESHWARI, S.C. 1980. Enhancement of pollen embryo formation in *Datura innoxia* by charcoal. *Physiologia Plantarum* **49**: 296 - 298.

UGENT, D. 1970. The potato. *Science* **170**: 1161 - 1166.

UHRIG, H. 1985. Genetic selection and liquid medium conditions improve the yield of androgenetic plants from diploid potatoes. *Theoretical and Applied Genetics* **71**: 455 - 460.

VASIL, I.K., AHUJA, M.R. and VASIL, V. 1979. Plant tissue cultures in genetics and plant breeding. *Advances in Genetics* **20**: 127 - 215.

VASIL, I.K. and NITSCH, C. 1975. Experimental production of pollen haploids and their uses. *Zeitschrift für Pflanzenphysiologie* **76**: 191 - 212.

VERGNE, P., DELVALLEE, I. and DUMAS, C., 1987. Rapid assessment of microspore and pollen stage in wheat and maize using DAPI and membrane permeabilization. *Stain Technology* **62 (5)**: 299 - 304.

WANG, C.-C., SUN, C.-S. and CHU, Z.-C. 1974. On the conditions for the induction of rice pollen plantlets and certain factors afffecting the frequency of induction. *Acta Botanica Sinica* **16**: 43 - 54.

WEATHERHEAD, M.A., BURDON, J. and HENSHAW, G.G. 1978. Some effects of activated charcoal as an additive to plant tissue culture media. *Zeitschrift für Pflanzenphysiologie* **89**: 141 - 147.

WENZEL, G. 1980. Anther culture and its role in plant breeding. *In* Symposium on Plant Tissue Culture, Genetic Manipulation and Somatic Hybridization of Plant Cells. Bhabha Atomic Research Centre, Bombay.

WENZEL, G. 1994. Tissue culture. *In* BRADSHAW, J.E. and MACKAY, G.R. (eds.), Potato Genetics, 173 - 195. Wallingford, UK. ISBN 0-851-98869-5.

WENZEL, G. and FOROUGHI-WEHR, B. 1984. Anther culture of *Solanum tuberosum*. *In* VASIL, I.K. (ed.), Cell culture and somatic cell genetics of plants, **1**: 293 - 310. New York: Academic Press. ISBN 0-127-15001-3.

WENZEL, G., HOFFMANN, F., POTRYKUS, I. and THOMAS, E. 1975. The separation of viable rye microspores from mixed populations and their development in culture. *Molecular and General Genetics* **138**: 293 - 297.

WENZEL, G., HOFFMANN, F. and THOMAS, E. 1977. Increased induction and chromosome doubling of androgenetic haploid rye. *Theoretical and Applied Genetics* **51**: 81 - 86.

WENZEL, G. and UHRIG, H. 1981. Breeding for Nematode and Virus Resistance in Potato via Anther Culture. *Theoretical and Applied Genetics* **59**: 333 - 340.

WERNICKE, W., HARMS, C.T., LöRZ, H. and THOMAS, E. 1978. Selective enrichment of embryogenic microspore populations. *Naturwissenschaften* **65**: 540 - 541.

WERNICKE, W. and KOHLENBACH, H.W. 1975. Anther cultures in the genus *Scopolia*. *Zeitschrift für Pflanzenphysiologie* **77**: 89 - 93.

WERNICKE, W. and KOHLENBACH, H.W. 1976. Investigations on liquid culture medium as a means of anther culture in *Nicotiana*. *Zeitschrift für Pflanzenphysiologie* **79**: 189 - 198.

WERNICKE, W. and KOHLENBACH, H.W. 1977. Experiments on the culture of isolated microspores in *Nicotiana* and *Hyoscyamus*. *Zeitschrift für Pflanzenphysiologie* **81**: 330 - 340.

WHITE, P.R. 1963. *The cultivation of animal and plant cells*, Second edition, 228. New York: Ronald Press.

WILSON, H.M., MIX, G. and FOROUGHI-WEHR, B. 1978. Early microspore divisions and subsequent formation of microspore calluses at high frequency in anthers of *Hordeum vulgare* L. *Journal of Experimental Botany* **29**: 227 - 238.

WOODCOCK, Kathryn M. and HOWARD, H.W. 1975. Calyx type in *Solanum tuberosum* dihaploids, *S. stenotomum*, *S. sparsipilum* and their hybrids. *Potato Research* **18**: 460 - 465.

YAMADA, T., SHOJI, T. and SINOTO, Y. 1963. Formation of calli and free cells in the tissue culture of *Tradescantia reflexa*. *Botanical Magazine, Tokyo,* **80**: 68 - 74.

ZAPATA, F.J., TORRIZO, L.B., ROMERO, R.O. and ALEJAR, M.S. 1982. Androgenesis in *Oryza sativa*. *In* FUJIWARA, A. (ed.), Plant Tissue Culture 1982, proceedings of the 5th International Congress of Plant Tissue and Cell Culture, 531 - 534. Tokyo, Japan.