

**AN EVALUATION OF POLLEN AS A VEHICLE FOR PLANT
TRANSFORMATION**

A thesis submitted for the degree of Doctor
of Philosophy at the University of Leicester

BY

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TO my parents for many years of encouragement
and prayers, and my wife for her support
and patience during the difficult times and
my brothers and sisters for their care and
concern throughout my study.

An evaluation of pollen as a vehicle for plant transformation.

Ph. D. Thesis, University of Leicester, October 1990.

Abstract

This thesis reviews the possibility of pollen grain/tube transformation and defines several problems which have hindered attempts to develop a routine technology for gene transfer. The importance of transforming pollen grains or using the pollen tube as path way for transformation has been discussed. As a prerequisite for transformation, the optimum conditions for pollen germination in vitro and fertilisation system with pre-germinated pollen were established for pea and tobacco.

Plasmid DNA (free or liposome-encapsulated) binding to the pollen tube was demonstrated using fluorescein and radioactive labelling techniques. The problems of introducing foreign genes into the pollen grain/tube and obtaining DNA integration and expression have been discussed. Selection techniques allowing potentially the detection of transformed pea and tobacco seedlings were developed.

Transformation methods involving using the use of naked DNA in vitro or in combination with PEG, the coating of pollen grain with plasmid, electroporation, microprojectile bombardment or the use of liposome-encapsulated plasmid were attempted.

Transformation methods in vivo using a 'pollen tube' pathway were attempted with tobacco, pea and wheat. DNA uptake by injection of *Agrobacterium* into pea florets was also attempted.

Although all of these transformation experiments reported in this thesis were unsuccessful it is still possible that the plant fertilisation system can be perturbed to allow gene transfer and in this respect an outline of possible future work has been given.

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ABBREVIATIONS

AP	Alkaline phosphatase buffer
Approx.	Approximately
AP STOP	Alkaline phosphatase stop buffer
BM	Brewbaker and Kwack media (1963).
bp	Base pairs
BAP	6-Benzylaminopurine
CMV	Cucumber mosaic virus
CaMV	Cauliflower mosaic virus
CAT	Chloramphenicol acetyltransferase
cm, mm, μ m	Centimetre, millimetre, micrometer
Cv	Cultivar
2,4-D	2,4-Dichlorophenoxyacetic acid
Da	Dalton
DAPI	4,6-Diamidino phenyl indol
dH ₂ O	Distilled water
Dicot	Dicotyledons
DMGT	DNA mediated gene transfer
DNA	deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
F ₁	First filial generation
FM	Franklin-Tong et al., media (1989).
g, mg, μ g	gramme, mlligramme, microgramme
GA	gibberellic acid
GB ₅	Gambourg B ₅ medium
GUS	Glucuronidase
HR	hour

IAA	Indole-3-acetic acid
I.M.S	Industrial methylated spirits
Kb	Kilobase pair
KD	Kilodalton
Km	Kanamycin resistance
l, ml, μ l	Litre, millilitre, microlitre
LUV	Large unilamellar vesicle
m, mM, μ M	Molar, millimolar, micromolar
Min	Minutes
MGR	Mean growth rate
MLV	Multilamellar vesicle
Monocot	Monocotyledons
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog media (1962).
M.W.	Molecular weight
NAA	Naphthalene acetic acid
NEO	Neomycin
No.	Number
NPT-II	Neomycin phosphotransferase
npt-II	Neomycin phosphotransferase gene coding region
nop	nopaline
oct	octopine
ori	origin
onc	Oncogenicity genes (Ti and Ri plasmid)
PEG	Polyethylene glycol
PMC	Pollen mother cell
REV	Reverse phase evaporation
Ri	root inducing
RNA	ribonucleic acid
RPM	Revolution per minute

SAAP	Streptavidine alkaline phosphatase
SDS	Sodium dodecyl sulphate
S	Sucrose media
sec	Second
SM	Simple media
SUV	Simple unilamellar vesicle
SV40	Simian Virus 40
TAE	Tris-acetate/EDTA
TBE	Tris-borate/EDTA
T-DNA	Transferred DNA
TMV	Tobacco mosaic virus
Ti	Tumour-inducing plasmid of <i>A. tumefaciens</i>
Tris	tris (hydroxymethyl)-aminomethane
tRNA	Transfer ribonucleic acid
μ	micron
U.V.	Ultraviolet
V, KV	Volt, kilovolt
v/v	Volume to volume
Vol	Volume
vir	Virulence gene (Ti and Ri plasmid)
w/v	Weight to volume
X-Gluc	5-Bromo-4-chloro-3-indolyl glucuronide

CHAPTER 1

1.0 GENERAL INTRODUCTION

Over the past decade, plant breeders have become increasingly interested in the potential of plant genetic engineering as an aid to conventional plant breeding. For example, the possibility of producing, herbicide-resistant plants (Shah et al., 1986; de Block et al., 1987) and insect-resistant plants (Fischhoff et al., 1987; Vaeck et al., 1987), virus resistant-plants (Tumer et al., 1987; Van Dun et al., 1987) and of improving quality of some seed crops, has led many large agro-chemical companies to make major investments into research on plant transformation.

There are two popular strategies for achieving gene

transfer into plant cells: *Agrobacterium*-mediated transfer and direct DNA transfer. A third strategy (indirect DNA transfer) has been adopted in the present work, in which DNA is to be introduced first into pollen and then into the sporophyte following fertilisation. The merits of each of these approaches have been discussed separately in sections 1.2 and 1.3 and for pollen as a vector in 1.8.1.

1.1 GENETIC ENGINEERING BY GENE TRANSFER

The genetic engineering approach to plant breeding aims to isolate and determine the structure and regulation of genes which control processes important in plant growth and productivity, and then to modify the existing gene, or to transfer new genes from other organisms, so that the performance of a particular plant is improved (Draper et al., 1988).

A common feature of all genetic manipulation techniques is that a gene receptor has to be combined with a gene donor. The gene receptor has to be a totipotent cell (the ability of a cell to express the phenotype of the whole plant), or a cell that has a clonal connection to the germline. Not all plant cells are totipotent and usually a very small minority of cells in plant tissues will be competent for both transformation and regeneration. Others will be competent for transformation or regeneration. A large fraction of the cell population will be potentially competent, which means that given the correct treatment they will have potential to shift to the competent state and a variable proportion of cells will not be competent (Potrykus, 1990). There are several

mechanisms by which a gene may be transferred from the gene donor to the gene receptor. These include passive plasmid uptake, *Agrobacterium*-mediated transfer, somatic hybridisation, virus infection and micro-injection. The transferred gene will, in some cases, be integrated into the recipient plant genome, expressed and most importantly genetically inherited. This technology broadens the possibilities of transferring genes between organisms (between plants, and from other organisms to plants) and creating novel genetic information by specific manipulation of genetic material to achieve the aims of a plant breeding programme. However, application of these techniques is often impeded by the lack of understanding of a genotype at the molecular level, including the gene products involved in the expression of a particular phenotype, as well as the unknown identity and character of the genes for most plant quantitative traits.

1.2 AGROBACTERIUM-MEDIATED TRANSFORMATION

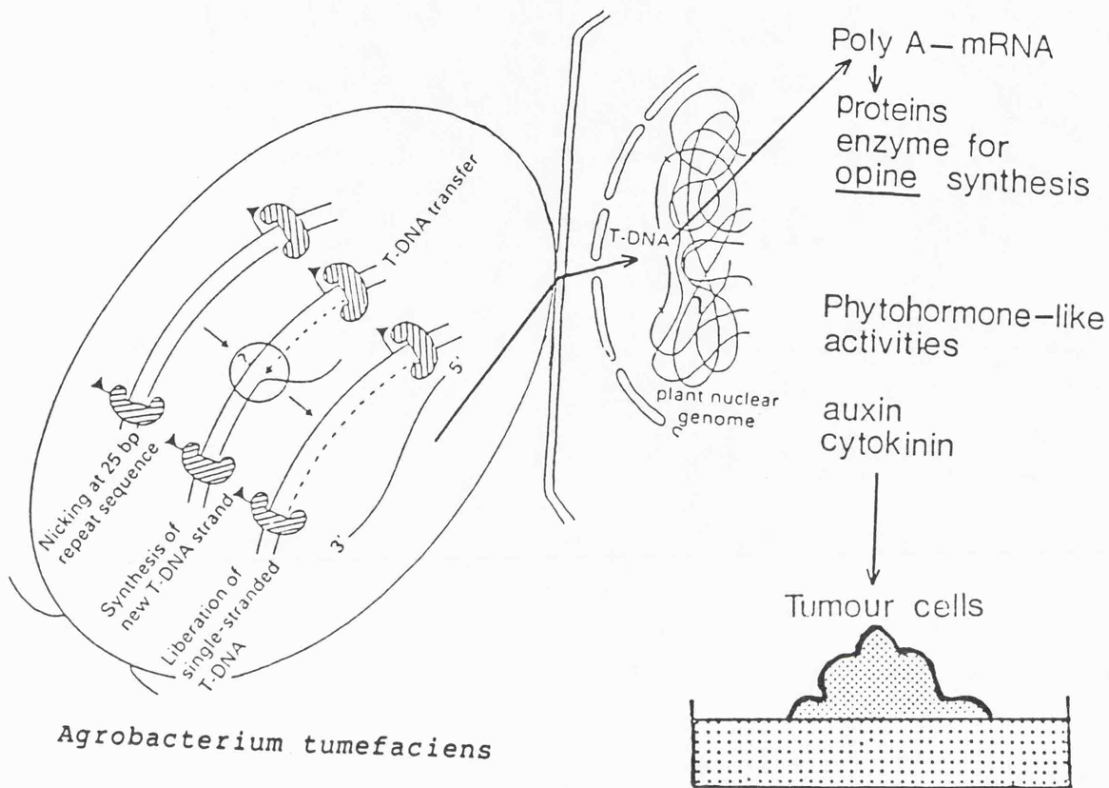
It has been widely recognized that a soil bacterium, *Agrobacterium tumefaciens*, carrying a Ti (tumour inducing), and *Agrobacterium rhizogenes* carrying a Ri (root inducing) plasmid provide the best naturally available systems for genetic transformation of higher plants. These plant pathogens induce respectively crown gall or 'hairy root' tumours after infection of a susceptible wounded host plant, by transferring and covalently integrating a defined segment of the Ti plasmid (T-Transferred DNA), into the nuclear genome of dicotyledonous cells at a wound site (Chilton et al.,

1977, 1980; Tepfer et al., 1984). With very few exceptions, T-DNA transfer has not been reported in monocotyledons, perhaps because of an absence of wound response (see Chapter 4; section 4.1). The T-DNA is expressed in plant cells (Drummond et al., 1977) and codes for variety of polyadenylated transcripts (Willmitzer et al., 1982; Bevan and Chilton, 1982). Some of these transcripts were shown to be responsible for the tumorous mode of growth of the transformed plant cells, while others code for enzymes that synthesize novel compounds called 'opines' (e.g. nopaline, octopine, mannopine), and can be the sole carbon and nitrogen source required for the growth of *Agrobacteria* (Fig. 1.1). The opine synthase genes provide useful markers for screening out transformed cell lines. The two major types of opines, octopine and nopaline, can be easily detected by a rapid and sensitive assay developed by Otten and Schilperoort (1978).

1.2.1 Tumour formation by *Agrobacterium*

The tumorous growth induced by T-DNA was shown to be primarily due to the activity of three genes, *tms1* (tumour morphology shooty), *tms2*, and *tmr* (tumour morphology rooty). The *tmr* locus encodes an enzyme involved in the synthesis of cytokinin, and mutations here result in root proliferation from crown galls induced on some species (rooty mutants). The *tms1* and *tms2* loci are involved with the unregulated synthesis of auxin, and mutations in either of these result in shoot proliferation (shooty mutant tumours) from crown galls on many types of plants (Draper et al., 1988). The synthesis of auxin and

Fig. 1.1 Mechanism of T-DNA transfer and tumour induction
by *A. tumefaciens*

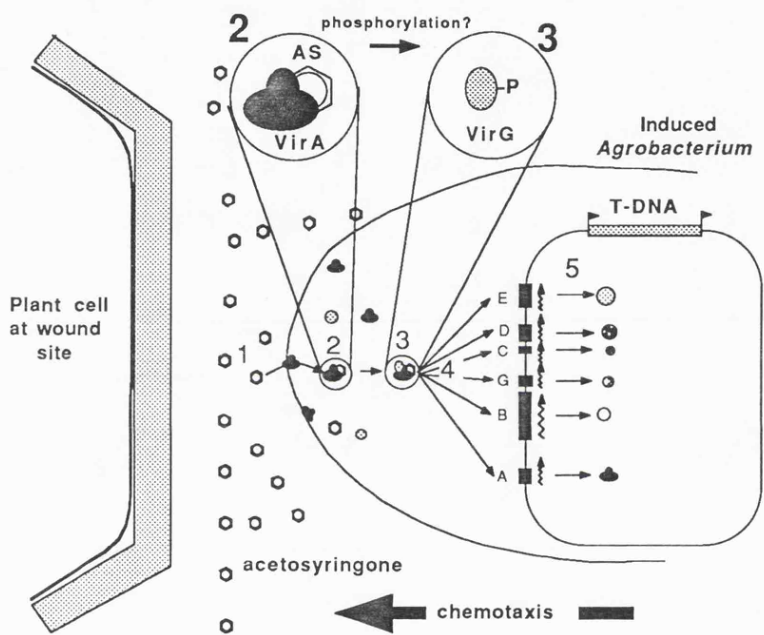


Growth of tumour cells on media lacking hormones

cytokinin by cells transformed with virulent *Agrobacterium* strains is an important consideration from the point of plant transformation/regeneration. A transformed cell expressing these genes will regenerate into an extremely abnormal plant, if it will regenerate at all (Klee and Rogers, 1989).

The T-DNA regions on all Ti and Ri plasmids are flanked by almost perfect 25 bp direct repeat sequence which delimit T-DNA on either end (Yadav et al., 1982; Barker et al., 1983). The right border of the T-DNA, is essential for transfer; deleting or reversing the orientation of the right T-DNA border abolishes T-DNA transfer, while manipulating the left border has little effect (Wang et al., 1984; Stachel and Zambryski, 1986). Although the T-DNA is the region being transferred to plant cells, no single mutation within the T-DNA abolishes its transfer; however, any mutation in a large section (approximately 35 Kb) called the virulence (vir) region of a Ti plasmid greatly attenuated or abolished tumorigenesis. So, it was suggested that this region was involved in T-DNA excision and transfer (see Fig. 1.1). T-DNA transfer could be achieved when the vir region was present in the same *Agrobacterium* cell on a separate plasmid (see Section 1.2.4), this indicated that the vir functions were trans-acting (Ooms et al., 1980; Stachel et al., 1985; Stachel and Nester, 1986; Horsch et al., 1986). The virulence region provides products required for plant cell recognition and T-DNA transfer and consists of six operons (vir A, B, C, D, G, and E; Fig 1.2). The function of several of these genes have been determined.

Fig. 1.2 Key events during the induction of the Ti plasmid *vir* genes by acetosyringone and other wound-related compounds: 1, acetosyringone released by plant cells at the wound site; 2, acetosyringone "activates" VirA protein located in the bacterial membrane; 3, activated VirA modifies VirG protein, possibly by phosphorylation; 4, modified VirG binds to the operator sequences of the *vir* genes resulting in their activation or regulation.



The 3' end of the nicked right border serves as a primary site for replacement strand synthesis to regenerate the bottom strand and a single stranded, T-strand is then released. The function of one of the vir E products (a DNA-binding-protein) could be in protecting the single-stranded T-DNA intermediate from degradation; the function of other vir region products are largely unknown. The vir B operon translation products have been identified in the bacterial cell envelope by Engstrom et al. (1987) who detected three vir B proteins (B33, B80 and B25) co-sedimenting with the cell envelope fraction; it was suggested that they may play a role in driving T-DNA transfer through the bacterial membrane.

1.2.3 Agrobacterium hosts

Wild type *Agrobacterium* strains contain so-called onc genes encoded by the Ti plasmid, whose expression results in plant growth regulator synthesis (auxin and cytokinin), and this in turn stimulates the cell division that leads to tumour formation. Several *Agrobacterium* strains have been 'disarmed' by having the onc genes removed after it was discovered that the 'onc' genes are neither required for the transfer of the T-DNA to the plant cell, nor its integration into the nuclear DNA (Draper et al., 1988). As has been stated earlier transformed cells expressing the 'onc' genes will regenerate into extremely abnormal plants, if they will regenerate at all (Klee and Rogers, 1989). On the other hand hairy roots induced by Ri plasmids have been shown to regenerate and have been exploited for transformation

(Tepfer et al., 1984). In general, the use of 'armed' strains is not recommended and should probably only be used in the absence of success with disarmed strains (Klee and Rogers, 1989). In disarmed strains with the onc genes removed this selective phenotype can be replaced by antibiotic-resistant genes offering resistance to antibiotics such as kanamycin.

1.2.4 Basic transformation vectors

The Ti plasmid has been exploited as a gene vector and a variety of useful Ti-plasmid-derived vectors and selectable markers have been constructed that allow for efficient plant transformation (Bevan, 1983, 1984; Fraley et al., 1983; Herrera-Estrella et al., 1983; Hoekema et al., 1983; Zambryski et al., 1983; Deblaere et al., 1985; Stachel and Zambryski, 1986). There are essentially two types of vectors that can be used for transformation, the cis-acting or cointegrative vector and the trans-acting or binary vector.

The cointegrative transformation vectors are plasmids that can not replicate in *Agrobacterium*. They can be maintained only by cointegrating into an endogenous plasmid, usually the Ti plasmid. For example, pGv3850 is a disarmed Ti plasmid, in which the T-DNA onc genes have been removed and replaced by a small cloning vector, pBR322. This is used in conjunction with an intermediate vector (based on pBR322) which contains a selectable marker gene (e.g. pGV1103; Hain et al., 1985). The vector is conjugated into pGV3850 and after cointegration the pGV3850::1103 T-DNA contains the whole of the intermediate

vector and the gene for transfer to plants is flanked by directly repeated bacterial plasmid sequences.

Binary vector systems are based on plasmids which replicate in both *E. coli* and *Agrobacterium* and use two compatible plasmids (for example pBin19; Bevan 1984). One plasmid lacks T-DNA but contains the *vir* region; a typical 'non-oncogenic' *vir* helper is pAL 4404 (Hoekema et al., 1983). The *vir* genes act in trans with another plasmid carrying a selectable marker gene, kanamycin resistance gene (neomycin phosphotransferase-II; NPT-II) which has been inserted into the binary plasmid between the left and the right borders. The *A. tumefaciens* strain harbouring both plasmids has a normal T-DNA transfer capacity, although neither is functional alone (for recent reviews see: Zambryski et al., 1989; Klee and Rogers, 1989)

Foreign DNA inserts of up to 50 Kb have been successfully transferred using these vectors. An upper size limit has not yet been determined (Herrera-Estrella et al., 1983). These transferred genes are expressed as dominant traits and essentially were inherited in a Mendelian manner (de Block et al., 1984; Horsch et al., 1984). The stability of T-DNA is critical to the commercialisation of transgenic plant technology. The number of plant species that can be transformed by *Agrobacterium* has been greatly increased (see Table 4.1; chapter 4), and now includes several of the most important crops (Draper et al., 1988; Gasser and Fraley, 1989 and Lindsey and Jones, 1989a).

1.2.5 Problems associated with Agrobacterium-mediated gene transfer

There are many reasons why an alternative system to Agrobacterium-mediated gene transfer should be sought. These are outlined below:

1.2.5.1 Host range

Perhaps the greatest weakness of the Agrobacterium system lies in its inability to transform most monocotyledons, especially the grasses which constitute the majority of the world's staple food plants. The reason for this host range limitation is still not fully understood (see Chapter 2; section 4.1), but may stem from the absence of a cell surface recognition site, acute sensitivity to Agrobacterium infection, or perhaps from an inability of one or more of the vir gene products to function. It should be emphasised that host range problems are not restricted to monocots. Indeed, many dicotyledonous species have not proved to be easy to transform using Agrobacterium.

1.2.5.2 T-DNA organisation

The integration site(s) of the T-DNA may not be suitable for radical manipulation and might impose a limitation on the application of T-DNA vectors. This could be particularly important if directed gene insertion is required as for example in gene replacement by homologous recombination. However, much more data on T-DNA organisation is required before it is possible to make a thorough assessment of this potential problem.

1.3 DIRECT GENE TRANSFER

A solution to the DNA manipulation problems and a partial answer to the host range limitation came with the successful transformation of plant protoplasts by direct uptake of vector DNA. Progress in protoplast transformation came with the advent of dominant selectable marker genes which were transcribed and translated efficiently in plant cells (Herrera-Estrella et al., 1983; Bevan et al., 1983). Access to relatively large amounts of vector DNA with selectable and/or screenable genes has generated much interest in DNA-mediated transformation. However, it should be emphasised, although it is now possible to transform protoplasts by direct gene transfer, obtaining regenerant plants from these cells is still highly problematical. Furthermore, until these regeneration problems are overcome, the transformation of protoplasts will have only limited applications. Techniques for uptake of nucleic acid into plant protoplasts are not new. They have been used for many years as a tool to study plant viral infection, replication and expression. Indeed, most current methods for DNA uptake into plant protoplasts were first developed for viral nucleic acid, which in turn were adopted from studies on the uptake and expression of viral genomes in cultured animal cells. It is particularly important to note that DNA Mediated Gene Transfer (DMGT) techniques could be used to transform both monocot and dicot protoplast. Chapter 4, outlines the various methods for DNA uptake into protoplasts, cells and pollen in detail. However, these techniques can be divided into two

categories. The first category is direct gene transfer methods which do not require protoplasts and work with other types of cells. Such procedures include microinjection (see Chapter 4; section 4.2.4); microprojectiles (see Chapter 4; section 4.2.5) and macroinjection (see Chapter 5; section 5.1.1.2). The second category contains methods which require protoplasts, and do not work with other types of cells, which includes polyethylene glycol treatment (see Chapter 4; section 4.2.1); electroporation (see Chapter 4; section 4.2.3) and the use of liposomes (see Chapter 4; section 4.2.6).

1.4 PLANT REGENERATION PROBLEMS ASSOCIATED WITH TRANSFORMATION

The aims of the majority of experiments using genetic transformation technology (*Agrobacterium*-mediated or direct gene transfer) is the regeneration of whole, fertile transformed plants and this regeneration, as mentioned earlier, is a result of totipotent plant cells in explants being given the correct hormonal and nutrient conditions.

Efficient plant regeneration can be achieved for many genotypes of various crops (for list, see Lindsey and Jones, 1989a). However, *in vitro* plant regeneration techniques often impose a degree of 'genome stress' especially if plants are regenerated via a callus phase. This stress often results in unwanted somaclonal variation. In contrast, transformed plants generated using techniques which do not perturb normal development should not suffer from such uncontrollable genetic

variability (Draper et al., 1988). Regarding transformation systems, in vitro regeneration is perhaps the main factor limiting yield and success (Heberile-Bors et al., 1990). Apart from organogenesis or embryogenesis from dedifferentiated cells, there are two other basic modes of development in tissue culture. Fig. 1.3 illustrates the three basic modes of explant growth in culture. It is appropriate to discuss the properties of possible 'target' cells in such explants following these different modes of development. A second objective will be to outline the problems associated with the transformation of dedifferentiated cells.

1.4.1 Dedifferentiation and shoot regeneration in cultured cells and somaclonal variation

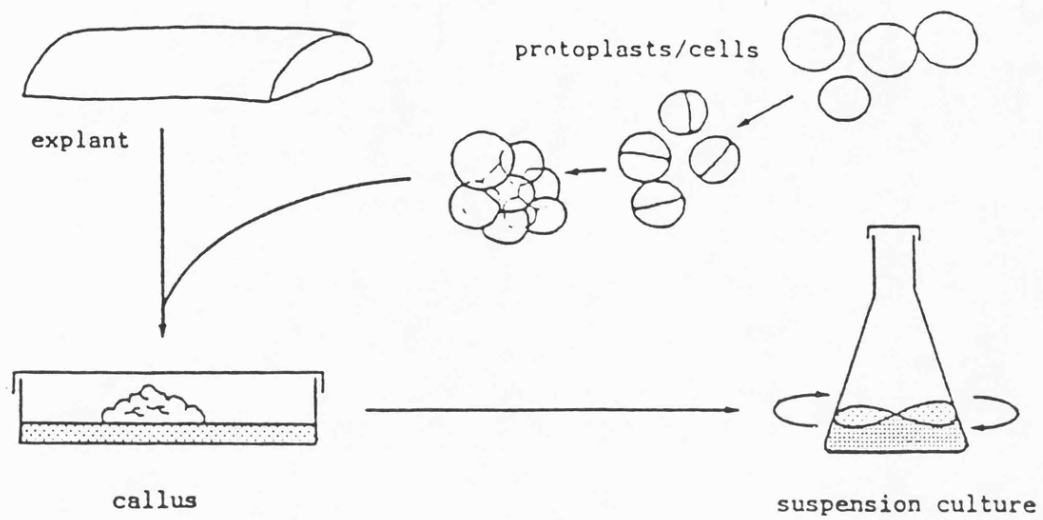
The source of dedifferentiated callus cells, also includes the development of cell colonies and callus from protoplasts or isolated plant cells. It is also possible to establish these undifferentiated tissues in a liquid suspension culture and grow them indefinitely.

1.4.1.1 Origin of adventitious shoots/plants

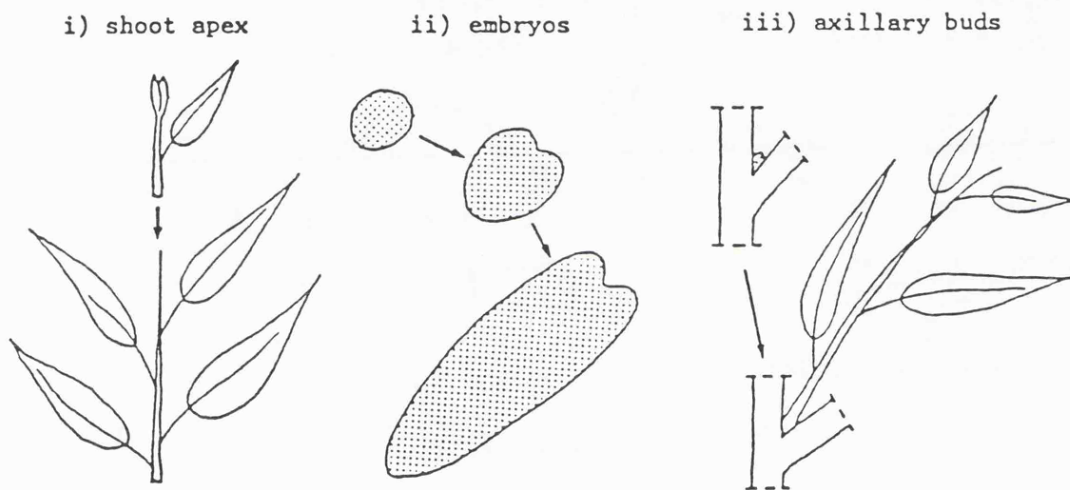
Whole plants can be regenerated from undifferentiated tissues in many species, either by organogenesis (shoot induction), or by embryogenesis. The adventitious shoots (developing via organogenesis) originate from a single cell within the callus and are, therefore nonchimaeric. In many species it is difficult to regenerate from protoplast-derived callus. Thus, if as in the case of most monocots, cultured cells are not

Fig. 1.3 Basic modes of growth in culture (from Draper et al., 1988)

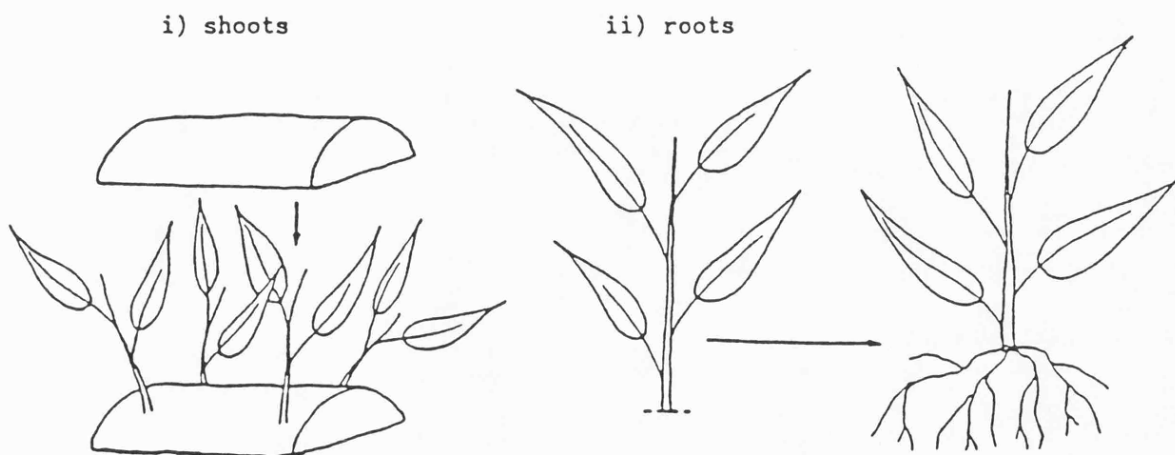
1) Undifferentiated



2) Development of existing organs



3) Development of adventitious organs



susceptible to *Agrobacterium* transformation then a gene transfer route is very difficult to develop.

1.4.1.2 Somaclonal variation in cultured cells

Cells in culture that pass through a callus phase can also develop many mutant trait changes normally referred to as somaclonal variation; this variation develops whether the regeneration occurs through somatic embryogenesis or by adventitious shoot formation. This includes plants regenerated from protoplasts, cultured plants (immature embryo, inflorescences, leaves, stems, tubers), microspores, anthers and ovaries, and also plants regenerated from cultured tumorous tissues (Lindsey and Jones, 1989b). Some of the causes of somaclonal variation are discussed in section 1.4.1.3.

This variation can be enhanced by the species and genotype, the source of the explant, the culture media used (in particular the plant growth regulators), and tissue culture procedure employed and time in culture. Changes in pollen fertility, flowering time, leaf weight, colour, size, shape, bud number, stalk length, absence of hair or plant height have been reported (Mantell et al., 1985; Draper et al., 1988; Lindsey and Jones, 1989b)

Somaclonal variation has been reported in many crop species such as oat, maize, barley, sorgham, onion, rape, lettuce, pelargonium and it is now evident that somaclonal can arise to some extent in most species that have been examined in detail after regeneration from callus phase (Scowcroft and Larkin, 1982; Mantell et al., 1985; Lindsey and Jones, 1989b). Somaclonal variation in potato has

been studied and agronomically useful experiments were achieved. For example some clones have been recovered which were resistant to early blight disease caused by *Alternari solani* (Matern et al., 1978). In another report by Shepard et al., (1982), 20 out of 800 somaclones tested proved resistant to late blight disease caused by *Phytophthora infestans*. However, in general when a gene is used to engineer a variety further uncontrollable genetic variation is probably undesirable.

1.4.1.3 Cause of somaclonal variation

Somaclonal variation is often associated with changes to the gross karyotype including cryptic chromosomal rearrangements, gene amplification or diminution and with transposable elements. The tissue culture environment may enhance the frequency of somatic crossing-over and sister chromatid exchange, and if a significant proportion of such exchange were asymmetric or between non-homologous chromosomes then genetic variants could be regenerated as a consequence (Scowcroft and Larkin, 1982 and Mantell et al., 1985).

The type of alterations to the nuclear genome of cultured plant cells and affect on regeneration have been reported (Baylisis, 1973; D'Amato, 1977; Constantin, 1981). There are essentially two categories of changes which affect the karyotype of plant cells kept in culture: Numerical changes and structural changes. Numerical changes to the karyotype involve an increase or decrease in the number of chromosomes in each cell. Whole sets of chromosomes can be added to the cell complement

(polyploidy), or else smaller numbers of chromosomes can be added or deleted from the cells (aneuploidy). Chimaeric plants may also arise in which cells in different parts of the plant have different chromosome numbers (agmatoploidy). Polyploidy is common in cultured cells and is thought to arise by endomitosis and endoreduplication. Aneuploidy is also common in cultured plant cells, although the precise mechanism leading to loss or addition of chromosomes is not fully understood. Numerical changes may arise as a direct result of the use of synthetic auxins such as NAA and 2,4-D in cultured media. These compounds are thought to affect spindle formation during mitosis (D'Amato 1977; Bayliss, 1973; 1980; Constantin, 1981; Mantell et al., 1985).

Structural changes to the karyotype are more cryptic and involve changes to the structure of individual chromosomes. The numerous ways in which such changes arise include peri-centric and para-centric inversions, translocation and somatic crossing-over. Structural changes are generally more difficult to detect than numerical changes, although the presence of laggards, chromosomal fragments and uneven separation of the chromatids is usually taken as fairly good evidence that structural changes may have occurred (Rieger and Michaelis, 1958).

1.4.2 Continued growth from pre-existing meristems

This method of development includes: a) The continuous growth of organs, like roots and shoots, as fully differentiated structures containing determined

cells; b) the continued normal development of immature structures such as early-stage embryos; and c) the development of quiescent axillary bud primordia into shoots. The problem with this system of regeneration is that it is difficult to target cells for manipulation since many cells give rise to the new shoot. Thus, any transformed cells may be lost in a chimaeric shoot. However, it may be possible to recover wholly transformed plants in the next generation. The advantage of this system is that less genetic variation is expected because of the absence of dedifferentiation.

1.4.3 Direct initiation and development of adventitious organ primordia

In this method shoots initiate from cells in original tissue explant without passing through callus stages. Adventitious shoots and roots are developed by the manipulation of growth regulators level in media. The problem with this type of growth that it is difficult to get access to such cells for physical DNA delivery or for *Agrobacterium* if the cells lie deeply within explant tissue. Also, no dedifferentiation occurs which makes it impossible to transform by *Agrobacterium*.

1.4.4 Summary of the problems associated with transformation and plant regeneration from tissue cultured cells

Cells which give rise to an adventitious shoot without a callus phase are often inaccessible either for *Agrobacterium* infection or for mechanical methods of DNA

delivery such as microinjection. Thus, although there are problems associated with mutation caused by cellular dedifferentiation, single cells and callus tissue are much easier to target.

Regarding chimaeric development problems, simply, one has to be able to transform the activated cell giving rise to the shoot primordia or the shoot will be chimaeric for transformed cells. However, there is a possibility of producing non-chimaeric seedlings from selfed seed of such a plant, provided at least some of the 'germline' tissues are transformed.

Protoplasts have many advantages for direct gene transfer, such as the absence of a cell wall greatly improves the probability of obtaining plasmid transfer and integration. When it is possible, whole plant regeneration from protoplasts usually derives from a single cell. Therefore, the regenerated transformants of such a system are unlikely to be chimaeric for the introduced gene. There are a wide range of techniques for introducing plasmids into protoplasts. Particularly important is the fact that DNA Mediated Gene Transfer (DMGT) techniques using plant protoplasts are not limited to a specific host range and so could be used to transform both monocot and dicot protoplasts. However, there are some disadvantages of using protoplasts for genetic engineering. Firstly, it is extremely difficult to obtain regeneration from protoplasts in most economically important species and as mentioned previously, there may be genome abnormalities induced by the regeneration process. All types of

transformation techniques (Electroporation, Micro-injection, Microprojectile and PEG) might also run the risk of decreasing the probability of achieving regeneration. Thus, perhaps pollen-mediated transformation is a viable alternative system. This project was initiated as no success had been obtained either *in vitro* using the same techniques which have been used for protoplast transformation or *in vivo* using the 'pollen tube' pathway.

1.5 BIOLOGY OF POLLEN

The possibility of using pollen grains as a vector for transformation will be presented in section (1.8.1). It is appropriate firstly to provide some important information regarding the biology and cytology of the pollen grain, pollen tube, pollen tube tip and the factors which affect the pollen germination *in vitro* such as the role of sugar, boric acid and pH.

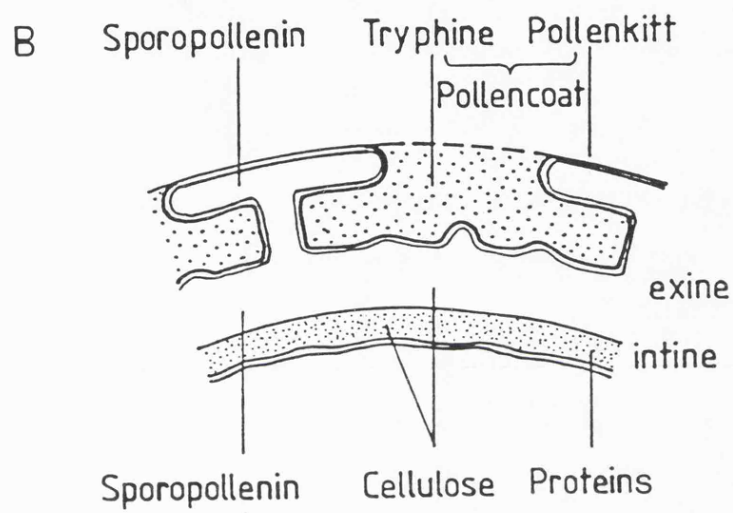
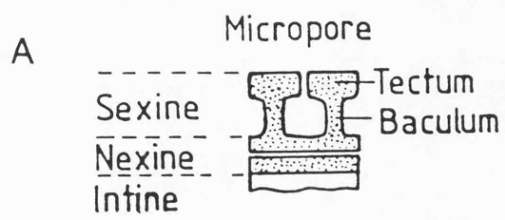
The pollen grain in angiosperms is a comparatively simple structure, it is the carrier of either two or three cells (one generative plus one vegetative, or two sperms cells plus one vegetative).

The pollen mother cell (PMC) undergoes a meiotic division to form a tetrad of haploid pollen grains. Shortly before the meiotic division, the primary walls of the PMC are replaced by thick layers of callose. In most cases the pollen grains of each tetrad are separated from one another by dissolution of the callose wall enabling them to lie freely in the pollen sac.

1.5.1 Pollen grain wall

The outstanding feature of the mature pollen grain is its wall, which is sculptured in a great variety of patterns, but at the same time within a species shows a high degree of consistency in organisation, chemistry and development. The wall typically consists of an exine and an intine (Fig. 1.3). The exine is either a uniform sheath or is subdivided into an outer layer (sexine) and an inner layer (nexine). Only the sexine is sculptured, it is attached to the nexine by means of rods or columns (the bacula), which may be united into a tectum above or remain free. In the aperture region, from which the pollen tube emerges, the sexine is rather sparsely sculptured and contains very short bacula (if present). The nexine becomes much thicker in the region of the aperture than in the rest of the pollen grain wall where it is discontinuous. The most distinctive chemical component of the pollen wall is the sporopollenin (Fig. 1.4), which consist of oxidative polymers of carotenoids and carotenoid esters (Shaw, 1971; Fahn, 1974; Knox, 1984). Sporopollenin is remarkably resistant to various chemicals, high temperature, and to agents which naturally decay organic matter (Crang, 1969). The intine is the inner polysaccharide layer of the pollen wall; the intine is remarkedly thickened and more complex in structure at the aperture where it forms the surface layer, it has a microfibrillar appearance and is synthesised by the microspore during the vacuolate period of development and contains cellulose, hemicellulose, and pectic polymers (Sitte, 1953; Ronald, 1971; Knox, 1983).

Fig. 1.4 Pollen wall comprises two layers: the exine and the intine. The exine commonly comprises also two layers: the sexine and the nexine (A). The exine which is the patterned layer and comprises the wall unit enveloped in the resistant polymer, sporopollenin (B). The exine covers the intine except at the germinal aperture (from Knox, 1984).



1.5.2 The male gametophyte

The mature male gametophyte consists of two or three cells resulting from one or two mitotic divisions of the microspores. These divisions take place inside the pollen grain. Prior to the first mitotic division the nucleus of the microspore takes up a position close to the wall. The first division results in the formation of two cells, the vegetative cell and generative cell. The generative cell initially has a callose or cellulose wall. Shortly after its formation the generative cell separates from the pollen grain wall and loses its callose wall (Mephram and Lane, 1970). It becomes surrounded by the cytoplasm of the vegetative cell and is often seen to be oval or lens-shaped. The division of the generative cell to two sperms is slow and in some plants, a spindle seems to be absent. The two sperms have large nuclei and the nucleus is surrounded by a layer of cytoplasm with mitochondria, dictysome, endoplasmic reticulum, and parallel arrays of microtubules (Standly and Linskens, 1974). The movement of the sperm cells is a result of cytoplasmic streaming (Navashin, 1969), and the streamlined shape of the generative cell permits it to move toward the tip within the central streaming region (van Went and Willems, 1984).

About 70% of angiosperms shed their pollen grains at the two-celled stage, while the remaining shed their pollen at the three-celled stage, after further mitosis of the generative cell has resulted in the formation of two male gametes.

1.5.3 Pollen cytology

The mature pollen grain contains a vegetative nucleus and a generative cell or two male gametes. The cytoplasm is also packed with mitochondria, plastids, golgi apparatus, ribosomes, starch grains, P(polysaccharide) particles, lipid droplets and protein stored within membrane vesicles of the endoplasmic reticulum.

1.6 BIOLOGY OF THE POLLEN TUBE

The pollen tube is produced from the aperture of the pollen grain after rehydration on the stigma, or in a suitable synthetic medium. The essential function of the tube emerging from the pollen grain is to deliver two male gametes into the embryo sac. The earliest observation of pollen tube growth outside the stigma appears to be that of Von Mohl (1834) who noticed that pollen grains of some species produced pollen tubes when placed in a saturated, humid atmosphere. Mature pollen grains are relatively quiescent, and have a latent metabolic system at the time of anther dehiscence. Uptake of the water *in vitro* and *in vivo* is an essential prerequisite for pollen tube development (Konar and Linskens, 1966)

Pollen of a number of species germinate readily in tap water and produce pollen tubes of considerable length because they contain small quantities of reserve food, usually in the form of starch, sugar, lipid and proteins (Vasil, 1960). The changes associated with germination are rapid and dramatic. The permeability of the plasmalemma of the vegetative cell is altered during

rehydration and at the time of germination (Shivanna and Heslop-Harrison, 1981). Water uptake and activation or synthesis of enzymes are the basic initiating factors for germination process (Rosen, 1971). A high rate of respiration is normally exhibited in *Lilium longiflorum*, the first phase occurring during the first 30 min coincides with rapid starch degradation. During the second period, from 30 until 60 min, respiration occurred at a lower rate. During the third period, from 60 to 120, the respiration rate increases and remains high thereafter. The higher respiration rate during phases 1 and 3 may be caused by increased ATP requirement for biosynthetic reactions such RNA, protein and membrane production. Turnover of ATP may decrease before tube initiation causing the low respiration for phase 2 (Dickinson, 1965). In vitro the enzymes are present in the pollen grain wall and readily diffuse out into the surrounding medium before pollen tube formation. Many of these enzymes are hydrolyzing, cell wall softening enzymes like β -1,3 and β -1,4-glucanase and pectinase (Stanley and Thomas, 1967). The enzymes help to soften the newly exposed intine in the region of the pore prior to pollen tube emergence. Free amino acids are also released rapidly when pollen grains are placed into a germination medium (Roggen and Stanley, 1969; Linskens and Schrauwen, 1969).

Before germination, important morphological events take place in the cytoplasm of *N. alata* pollen. The most evident changes are: the setting free of the rough endoplasmic reticulum cisternae from the stacks present in the mature pollen and their interaction with lipids; the

considerable activity of dictyosomes; the aggregation of the ribosomes to form polysomes; the formation of lamellae and starch grains in the plastids; and the gradual disappearance of the fibrillar masses. The activation of protein synthesis is probably from RNA stored during pollen development in a stable form in order to ensure a quick resumption of synthetic activity on pollen germination. The vegetative nucleus and the generative cell do seem to undergo significant changes in comparison with the inactive mature pollen (Cresti et al., 1985). During the first minute alone, about 50% of the free amino acids leave the pollen grain (Vasil, 1987).

The distribution of organelles in the cytoplasm tube of *Lilium* pollen was not uniform with an increasing abundance of mitochondria, amyloplasts, Golgi bodies, endoplasmic reticulum, lipid bodies, and vesicles and mitochondria and dictyosomes towards the tip (Rosen et al., 1964). In the newly-emerged and elongating pollen tube Golgi-derived vesicles accumulate in the tip of the newly-formed pollen tube to the virtual exclusion of all other cell organelles. These help delimit the growing region of the pollen tube forming the cap block (Vasil, 1987).

The formation of the new wall and cell membrane occurs in the tip of the tube by the activity of the secretory vesicles, which are probably produced by the dictyosomes located in the subapical zone. The secretory vesicles fuse with the plasma membrane at the tip of the tube; the vesicle contents are discharged outside and contribute to the new cell wall formation, while the

vesicle membranes form the new plasma membrane (Steer and Steer, 1989). Active transport of the vesicles may occur through the cytoplasmic membrane system and may be involved with the progressive disappearance of fibrillar masses. These masses, possibly composed of actin, disperse during germination and could provide the cytoskeletal structure upon which cytoplasmic streaming and sperm cell migration occur (Cresti and Went, 1976).

The "non-growing" and mature region of the pollen tube contains a granular and vacuolated cytoplasm with an abundance of mitochondria, amyloplasts, golgi bodies, smooth and rough ER, lipid bodies, and vesicles of various types. The pollen tube wall is usually about 0.1 μm thick and consists of three layers (Rosen et al., 1964). Older pollen-tube walls contains three layers, whether in the style or in vitro. The wall layers are, (1) an outer microfibrillar pectic coating; (2) a microfibrillar, mainly cellulosic, middle layer; and (3) an inner callosic sheath (Heslop-Harrison, 1979b).

1.6.1 The pollen tube tip structure and its activities

The tube tip is the only part of the pollen tube which can grow. The tip wall has frequently been described as being pectic in nature with the callose and cellulosic component of the mature tube wall being absent and the thickness of the wall at the tip in in vitro-grown tubes is extremely variable, and under optimal growth conditions the wall is 50 - 100 nm thick. Transferring pollen tubes to less favorable conditions can lead to a rapid increase in wall thickness at the extreme apex and

cessation of growth (see Chapter 4; section 4.4.2).

The tip cytoplasm is most unusual in its composition. The normal matrix background of cytoplasmic ribosomes is absent. Ribosomes are present in only small numbers scattered in a darkly staining matrix of fibrils, about 3 - 7 μm in diameter. The mass of fibrils is assumed to be part of the cytoskeleton (Steer and Steer, 1989). The only organelles in the tip are the secretory vesicles. The growing tip is characterised by abundance of vesicles. The vesicles appear to be of 2 types. One is spherical, about 0.1 μm in diameter, apparently arises from the Golgi apparatus and appears to contribute to tube wall and plasmalemma formation. The other type is irregular in shape, 0.02 - 0.05 μm in diameter, and is of unknown origin and function. Cytochemical analysis indicates that the tip of *Lilium* pollen tubes are singularly rich in ribonucleic acid, protein, and carbohydrate (Rosen et al., 1964). The membrane of the secretory vesicles is a product of the endomembrane system of the tube, destined for incorporation into the plasma membrane following vesicle fusion with the apex (Brawley and Robinson, 1985).

The pollen tube tip is a site of intense activity, involving the generation of plasma membrane and new cell wall from the considerable flow of vesicles to the apex. At the molecular level there must also be a highly active cytoskeleton in the tip cytoplasm and active ion pumps and channels in the plasma membrane (Steer and Steer, 1989).

1.7 IN VIVO AND IN VITRO POLLEN GERMINATION

Pollen tube cytology and physiology may differ under artificial and natural conditions. There are also important physiological difference between 2- and 3-celled pollen grain germination (see Chapter 3; section 3.1)

1.7.1 Cytoplasmic streaming

Cytoplasmic streaming becomes apparent in pollen grains minutes after they are placed in a suitable environment for germination. Rapid streaming takes place in the tube during its growth and continues for a while even after elongation has stopped (Vasil, 1960; Mascarenhas and La Fountain, 1972). The rate of streaming varies not only from plant to plant, but is different in different parts of the pollen tube (Vasil, 1987) and the rate of protoplasmic streaming changes with the elongation of the tube (Iwanami, 1956). In earlier stages of growth, the protoplasm of the pollen tube streams towards the tip along the wall of tube and runs back from near the cap-block. In the centre of the tube movement occurs in the reverse direction. From this time onwards the pollen tube becomes somewhat attenuated except near the tip. Because of the appearance of the vacuoles in the pollen grain and the tube, the protoplasm now streams in the form of a narrow band along the side of the tube. The movement towards the tip is along one side of the tube while the protoplasm travels back to the grain from the other side. However, near the tip of the tube streaming is in the usual way (Iwanami, 1956). In general, the rate of

streaming is slow towards the grain end of the pollen tube, is fairly rapid in the rest of the tube and there is no streaming in the cap block at the tip of the tube (Vasil, 1987).

As cytoplasmic streaming circulates and rotates different organelles around the tube, its importance comes from its ability to drive internalised exogenous DNA or liposomes towards the generative cell or the two sperm cells to the ovule.

1.7.2 Callose plug

The growing pollen tubes *in vitro* or *in vivo* are not open pipes, but closed with callose plugs. The first callose plug is usually formed at a fixed distance from the tip of the pollen tube; this distance varies between species and possibly between populations. This position is usually after the tube has reached a length of 1000 μm or more, and after the nuclei and most of the cytoplasm from the pollen grain have moved into the pollen tube (Vasil, 1960). The plug starts as a narrow and continuous circular band of thickening on the tube wall, or it may be initiated as localised growth on one side of the wall and then extend to the other side (Vasil, 1960, 1962). In artificial culture media, where optimal conditions for growth are often not present, much more callose accumulates than occurs in a normal compatible pollination in nature (Vasil, 1987). Thus, introducing DNA or liposomes *in vivo* or *in vitro* can be attempted only before callose plug formation.

1.7.3 Role of sugar (sucrose)

Pollen grains contain some reserve food materials at the time shedding which are used as energy for germination. Such reserves may be in the form of fixed oils, sugars or starch (Baker and Baker, 1979). However, these reserves are not adequate for the enormous growth of the pollen tube involving the synthesis of large amounts of cell wall material, which takes place *in vivo* and *in vitro*. Sugar supplied to the germinating pollen in culture supports sustained growth and in some cases results in pollen tubes that are as long as those produced *in vivo*. Pollen grains of a wide range of taxonomically distant species germinate successfully in sugar solution. Sucrose is probably the best and most commonly used source of carbon and energy for pollen (Vasil, 1987), although several other sugars such as glucose, galactose, lactose, dextrose, rhamnose and raffinose have also been found useful, while fructose, mannose and mannitol are mostly unsatisfactory (Stanly, 1971; Vasil, 1987). Sucrose had the most marked effect on the respiration of pollen tubes (Hrabertova and Tupy 1963 and Tupy, 1961). It appears to be probable that the primary factor in the specific effect of sucrose and raffinose is the presence of bond β -D-fructofuranose in the sugar molecule (Hrabetova and tupy, 1963). Significantly the enzyme β -fructofuranosidase has been shown to be present in pollen (Dickinson, 1967). Sucrose also plays an important osmoregulating function in most pollen germination media.

1.7.4 Role of boron (boric acid)

Boron is an essential element required for the normal growth and development of higher plants. Flowers, especially the tissues of stigma, style and the ovary, often contain high concentrations of boric acid. The excessive bursting of pollen grains and pollen tubes so often encountered in the absence of boron from the nutrient media may be due to a close negative correlation between tissue hydration and the supply of boron, and retarded deposition of new wall materials in the growing tip of the pollen tube (Gauch and Dugger, 1954). Boron encourages sucrose absorption prior to the stimulation of pollen tube growth. It is therefore possible that the stimulatory effect of boron on growth is connected with carbohydrate metabolism (Tupy, 1960).

1.7.5 Effect of pH

Pollen germination has been reported in media with pH values between 3.5 and 9.5, although optimal levels in media containing boric acid are generally between pH 6.5 and 7.1 (Stanley and Lichtenberg, 1963). Vasil and Bose (1959), observed that the pH of the medium was virtually unchanged, even after two hours pollen tube growth.

1.8 THE PROJECT AND ITS AIMS

1.8.1 Pollen as a Vector

Heslop-Harrison (1979a) rightly termed the gametophytic portion of the angiosperm life cycle the "forgotten generation". This neglect, however, came in

spite of a constant indication that pollen is the site of intense gene activity and selection (Mulcahy, 1985).

DNA introduced into pollen grains or the pollen tube subsequently should be taken theoretically to the ovule where transformation of gametes or zygotes may be possible. If it occurs, such an approach would be technically simpler, faster and universal in its application than other transformation methods (Heberle-Bors et al., 1990; see section 1.8.2).

Transformation using pollen as a vector was proposed in the early 1970's (see Chapter 4; section 4.3; Hess, 1974, 1975; Wanger and Hess, 1972), when the difficulties with cereal protoplast regeneration had just become apparent, but it is still not developed as a routine technology (see Chapter 4; section 4.1). Gene transfer via pollen would be very attractive but has failed to materialise, the problem is that few molecular biologists have a wide knowledge of pollen development and few pollen physiologists have a working knowledge of transformation systems. Therefore all of these early attempts failed to use sensible dominant marker genes to recognise transformants (see Chapter 4; section 4.3).

1.8.2 Advantages of using pollen as a vector

Pollen offers readily available target cells for direct gene transfer. In some species pollen is amenable to work with, it can be collected in large quantities and can be stored for relatively long periods of time. Pollen mediated gene-transformation is not necessarily restricted to the dicotyledons and additionally, transformation

systems involving pollen will avoid alteration to the nuclear genome which are associated with tissue culture and regeneration. A pollen based transformation system reduces the chances of genetic chimaery, since transformants are all derived from a single cell (the fertilised egg cell); thus, once developed, transformation techniques will probably be cheap and quick because there is no need for tissue culture.

1.8.3 Disadvantages of using pollen as a vector

There are potential disadvantages of using pollen as a vector. For example, in any pollen transformation system, only a limited number of the pollen population actually used, would be possibly transformed. Therefore, a pollen-mediated transformation system will require large scale screening or selection of the progeny. The fitness of pollen to effect fertilisation may be reduced by plasmid uptake. Additionally, introducing foreign genes into the pollen, (or the ovule) might disrupt the fertilisation process.

1.8.4 The aims

The main aim of this thesis is to assess the feasibility of introducing foreign genes into plants via the transformation of the pollen in three species, pea (*Pisum sativum*), tobacco (*Nicotiana tabacum*) and wheat (*Triticum sativum*)

There are 5 interim objectives to be realised before pollen-mediated transformation can be realistically attempted:

- (1) To establish a reliable *in vitro* pollen germination system for both species.
- (2) To obtain and optimise fertilisation from pollen germinated *in vitro*.
- (3) To establish a mechanism of DNA entry into pollen.
- (4) To obtain seeds by fertilisation with DNA-treated pollen.
- (5) To test and monitor genes in transformed pea callus and develop conditions that should allow the selection of transformed seedlings.

Thus, the success of the project depends on the development of an experimental system which introduces plasmid DNA into a high number of pollen grains or pollen tubes without impairing their capability of fertilising the egg-cell.

As mentioned earlier, two species have been chosen for this project tobacco and pea. They both have bicellular pollen which is amenable for storage and *in vitro* germination, they produce large amounts of pollen and their seeds are easily to germinate and the seedlings are easy to grow. Tobacco has been a model system in many genetic engineering experiments. Tobacco flowers contain a high number of ovules per ovary which has a potential for producing many seeds. Its anther is accessible and contains copious quantities of pollen and is also positioned at the end of a corolla tube which is easily to remove. The tobacco ovary has a large loculus into which it may possible to inject plasmid or treated pollen. Tobacco plants have a relatively short generation

time and long flowering period. Pea is a commercially important species which has yet to be transformed. It has a short generation time and its ovary has a moderately sized loculus. Transformation of pea using *Agrobacterium* has been reported (Hussy, et al., 1989 and Hobbs, et al., 1989), and also by DNA associated liposome in pollen grains (Ahokas, 1987). However, in none of these reports were transgenic plants described and fully characterised.

CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 PLANT MATERIALS

The tobacco (*Nicotiana tabacum* L) variety SR1 and Wheat of variety Timmo and two leafless varieties of pea (*Pisum sativum* L; cultivars Consort and Orb) were used in this study. In addition to these pea cultivars FS4 and Countess were used in transformation experiments using *Agrobacterium*. BBB Seeds were sown in John Innes compost and the seedlings were grown to maturity under 400 watt high power sodium lamps in a heated glasshouse. The glasshouse temperature varied between 14 and 24°C.

2.2 PLANT PREPARATION

Tobacco anthers from mature flowers and pea anthers

from the 1st, and 2nd, or 3rd mature flower of an individual plant were collected shortly after dehiscence. These were then ruptured with fine forcep to release their pollen into Universal bottles containing 1 ml of germination medium. Mature wheat pollen was collected and treated as for tobacco pollen.

2.3 POLLEN GERMINATION MEDIA

The germination media used for tobacco and pea pollen, all contained 0 - 40% sucrose (usually 15%) and 0 - 500 mg/l boric acid (usually 100 mg/l).

The media used for tobacco were as follows:

- S containing only sucrose.
- SM (simple or basic simple) containing only sucrose and boric acid.
- SK containing sucrose, boric acid and 100 mg/l K_3PO_4 .
- SKM containing sucrose, boric acid and 100 mg/l K_3PO_4 , 100 mg/l $Mg(NO_3)_2$.
- BM containing mineral salts according to Brewbaker and Kwack (1963), sucrose and boric acid.
- FM containing mineral salts according to Franklin-Tong et al., (1989), sucrose and boric acid.

The media which were used for pea were: SM, BM, and FM.

2.4 IN VITRO GERMINATION OF POLLEN

For most experiments the pH of the medium was set to pH 6.5 for pea and 6.75 for tobacco using NaOH/HCl or Tris HCl; the pH of the medium was re-checked at the end of each experiment. Samples were usually incubated in a

controlled environment room at 25°C under fluorescent illumination for 2 - 24 hr, to allow germination. 20 - 50 μ l aliquots were taken at regular time intervals and examined under the microscope (x 10) to score percent germination and tube length. At least 200 grains were used to score percent germination in each aliquot. A pollen grain was judged to have initiated germination only when tube length exceeded grain diameter. Burst tubes were counted as ungerminated. The lengths of twenty randomly selected unburst pollen tubes were measured from each aliquot. For tobacco, tube growth and percent germination were recorded after 4 hr. The pea pollen was germinated in incubation density of 6000 - 27000 grains/ml and for tobacco, 6000 - 27000 grains/ml. A student t-test or analysis of variance (one-way classification) was applied to test for differences between pollen growth or percentage germination in different media, genotypes, replicates or samples.

2.5 FERTILISATION PROCEDURES WITH PRE-GERMINATED POLLEN

2.5.1 Germination in liquid media

Pollen was germinated in 1 ml of liquid media and spun down at low speed (6500 rpm) for 20 - 45 sec. 0.75 ml of the supernatant was discarded and the pollen was resuspended in the remaining 0.25 ml. A small quantity of the concentrated pollen suspension was then transferred to stigmata.

2.5.2 Germination on cellophane

A small piece of cellophane (approximately 1 cm²)

was placed over 200 μ l of liquid medium. Pollen was dusted onto the cellophane, and placed in a humid atmosphere at 21°C for 1 - 3 hr to allow germination. The piece of cellophane was then dipped in liquid medium, shaken a few times to release the pollen, then treated as for the germination in liquid medium.

2.5.3 Germination in semi-solid media

Pollen was applied to a liquid medium which was then supplemented with 300 - 400 mg (per 100 ml media) of Seakem agarose to produce a semi-solid consistency. The pollen was then incubated at 21°C for 1 - 3 hr to allow germination. More liquid medium was added to dilute the semi-solid medium and then the pollen was spun down for 20 - 45 sec, and then treated as for the germination in liquid medium.

2.6 PLANT TRANSFORMATION PROCEDURE

2.6.1 Bacterial culture

In sterile conditions, a single colony of *Agrobacterium* was picked from a fresh bacterial plate containing selective antibiotics, transferred using a flamed and cooled loop to 5 ml of nutrient broth (see below) containing a suitable selective antibiotic in a Universal bottle. The bottle was incubated on an orbital shaker (150 rpm) for 2 days at 28°C.

Oxoid nutrient broth

13 gm of oxoid nutrient (Lot No. 070 380 68. made by Oxoid Ltd., Basingstoke, Hants., England), was dissolved in 1.0 L of distilled water, mixed well and sterilised by

autoclaving at 121°C for 15 min.

The oxoid nutrient broth contains in 10 L:

'Lab-Lemo' Beef extract (oxoid L29)	1.0 gm
Yeast extract (oxoid L20)	2.0 gm
Peptone (oxoid L37)	5.0 gm
Sodium chloride	5.0 gm
pH (approx. 7.4)	

2.6.2 Agrobacterium strains

One oncogenic strain of *A. tumefaciens* and two oncogenic strains of *A. rhizogenes* were used in this project, provided kindly by Mr D. Firn.

Agrobacterium strain	antibiotics ($\mu\text{g/ml}$)
A281	50 $\mu\text{g/ml}$ kanamycin
	50 $\mu\text{g/ml}$ rifampcin
AG84	50 $\mu\text{g/ml}$ kanamycin
9402	5 $\mu\text{g/ml}$ tetracycline
9402 (pBI.121.1)	50 $\mu\text{g/ml}$ rifampcin
	50 $\mu\text{g/ml}$ kanamycin
A281 (pBI.121.1)	50 $\mu\text{g/ml}$ rifampcin
	50 $\mu\text{g/ml}$ kanamycin

2.6.3 Inoculation of pea stems

The pea stems were excised from 14-day old seedlings from greenhouse grown plants. The stems were surface sterilised with 10% Domestos for 20 min, rinsed three times with a sterilised tap water, then they were placed on a sterile white tile, and cut to small segments (4 - 6 cm). The segments were placed onto a 9 cm plastic Petri dish with 25 ml of agar-solidified with 0.9% agar media of

MS, then the stems were stabbed with a needle (25 gauge), previously dipped in 10x dilution an overnight culture of *Agrobacterium*. The inoculated stems were incubated at 25°C at a light density (3000 lux.). Control (stabbed with a needle) stems were treated in the same way.

2.7 TISSUE FIXATION

Styles were fixed in 3:1 ethanol:glacial acetic acid for 3 hr and then incubated overnight in 8 M NaOH at 60°C to soften the tissue. Styles were then washed in DH_2O and stained in 0.2% aniline blue to visualise the pollen tube walls. The styles were squashed under a cover slip and then examined under the U.V. microscope.

2.8 POLLEN FIXATION

The pollen of each tube was spun down for 30 sec and the pellet resuspended in 50 μl of medium. About 5 μl of pollen suspension was placed on a slide, 40 μl of Q water was added to burst the tubes and release the cytoplasm. The slides were put onto a slide heater (40 - 50°C) in a flow hood to prevent dust setting on the slides. When the slides were dry they were carefully placed in 3:1 (ethanol:glacial acetic acid) for 40 min and then passed through an ethanol series 50% 70% 95% ethanol 10 min each in Coplin jars, the slides were air dried could be stored in foil at 4°C overnight if required.

2.9 NUCLEASE ACTIVITY

The content of one anther was released in 100 μl of germination media in the presence of 50 μl (300 $\mu\text{g/ml}$) of

pCaMVNEO for different incubation times (10, 15, 20 min). The pollen was centrifuged for 10 sec and 40 μ l of the supernatant was run in a gel. To determine the effect of washing on the nuclease activity, the experiment was repeated but the media was replaced with fresh media after different times (15, 20, 25 min), and 5 μ l of plasmid was added to the washed medium, then 40 μ l was run in a gel.

2.10 HISTOCHEMICAL LOCALIZATION OF GUS

Samples of pollen were placed in fixation solution (0.3% formaldehyde, 10 mM MES pH 5.6, 0.3 M mannitol), vacuum infiltrated briefly (1 min) and then incubated in fixative for 45 min at room temperature, followed by several washes in 50 mM NaPO_4 pH 7. The pollen was transferred to 1-2 ml of histochemical reagent (2 mM 5-bromo-4-chloro-3-indolyl glucuronide) (X-GLUC) and incubated at 37°C for 2, 5, 24 and 84 hr. After staining, the sample was rinsed in 70% ethanol for 5 min and then mounted on microscope slides for microscopy.

2.11 CHOICE OF VECTORS

Four plasmids were chosen as plant transformation vectors for this project, pBI.121, pBI.221 (Jefferson et al., 1987), pCaMVNEO (Fromm et al., 1986), and pCaMVCAT (Fromm et al., 1985). Plasmid pBI.121 is 12.8 kb and contains a nos-npt-II gene and additionally the β -glucuronidase (GUS) gene under the control of the CaMV 35S promoter. Activity of this latter gene in transformed tissue can be easily detected using of a number of assays. The pBI.121 plasmid is characterised by its size and by a

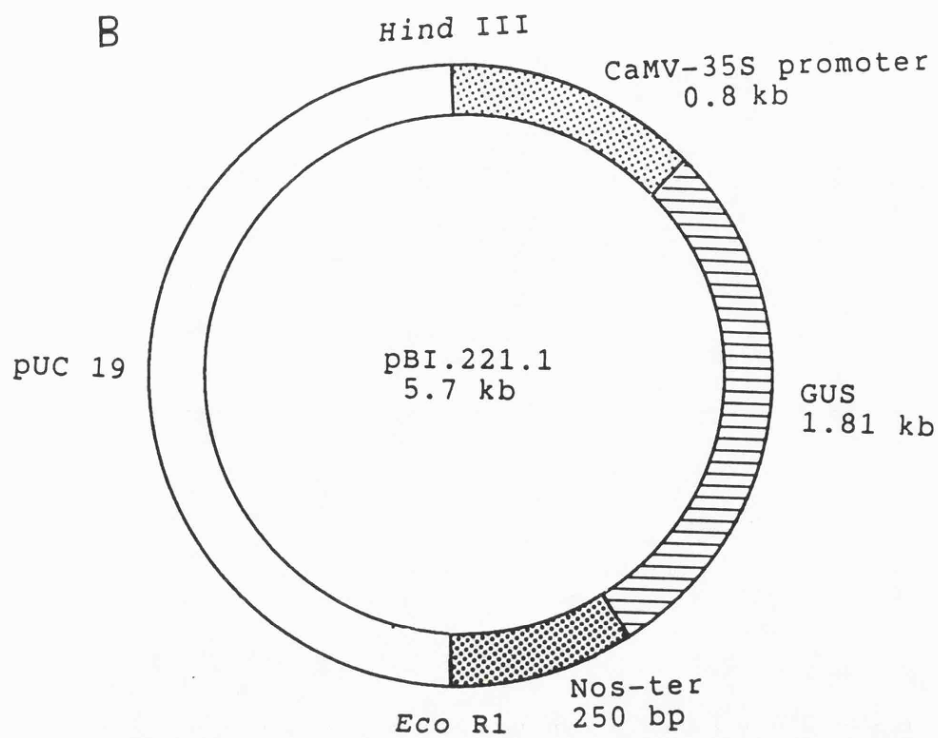
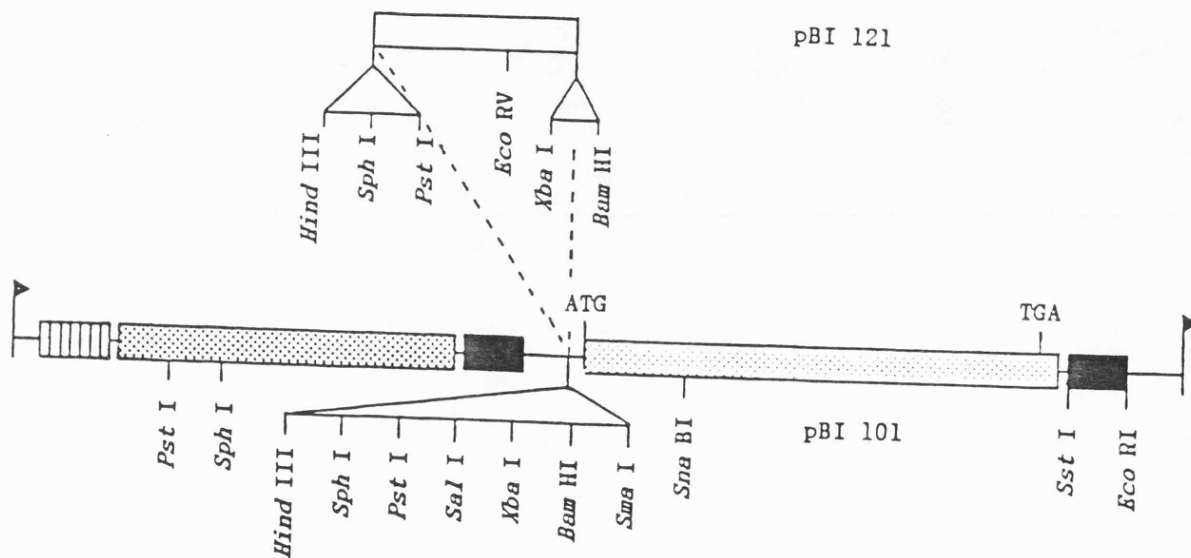
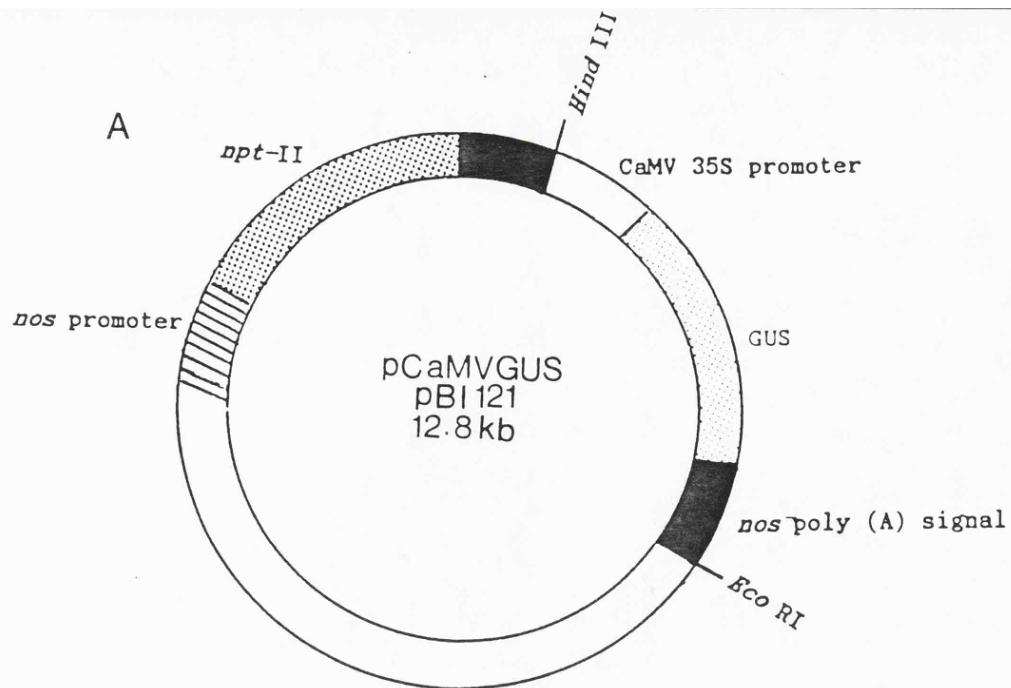
Hind III and a *Eco* RI restriction site which divides the plasmid into 10 kb and 2.8 kb fragments (Fig. 2.1 A). The pBI.221, was used for microprojectile experiments with pollen and for electroporation experiments (Fig. 2.1 B). The pCaMVNEO can be recognised on the basis of its size (4.4 kb) and by its two *Hind* III and *Bam* HI restriction sites (Fig. 2.1 C). It contains the neomycin phosphotransferase II (npt-II) gene which is a useful dominant marker that confers resistance to the antibiotics kanamycin and G418. The antibiotic resistance gene is under the control of the powerful cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase polyadenylation signal. The pCaMVCAT plasmid was used in early attempts to transform wheat, it contains the screenable/selectable marker chloramphenicol acetyl transferase (CAT; Fig.2.1D).

2.12 LARGE-SCALE PLASMID ISOLATION FROM *E. COLI*

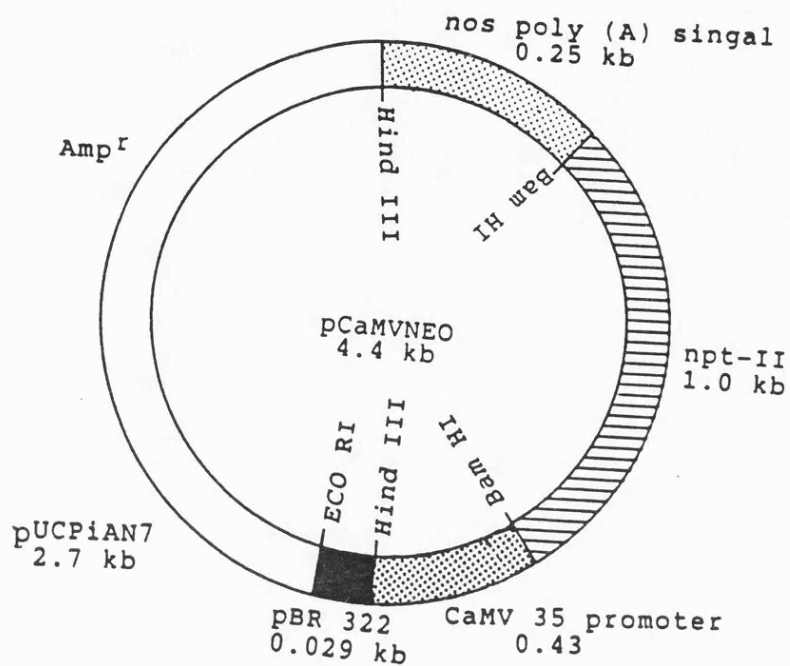
A single colony of *E. coli* which contained an appropriate plasmid was picked into 5 ml of NB containing selective antibiotics and shaken for 6 hr, or overnight. 50 μ l of this starter culture was subcultured into 500 ml NB containing kanamycin in a baffled 2 litre flask and shaken overnight at 37°C. Bacteria were harvested by centrifugation in 2x250 ml bottles at 8000 rpm and 4°C for 5 min, using a Sorval RC-5B (or equivalent). The supernatant was poured off and the pellet was resuspended in 2x20 ml of lysis solution (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5 M glucose, 1 mg/ml lysosome) then, they were pooled and incubated on ice for 5 min. 80 ml of alkaline SDS (0.2 M NaOH, 1% SDS) was added, mixed very well and

Fig. 2.1 Structure of plasmids

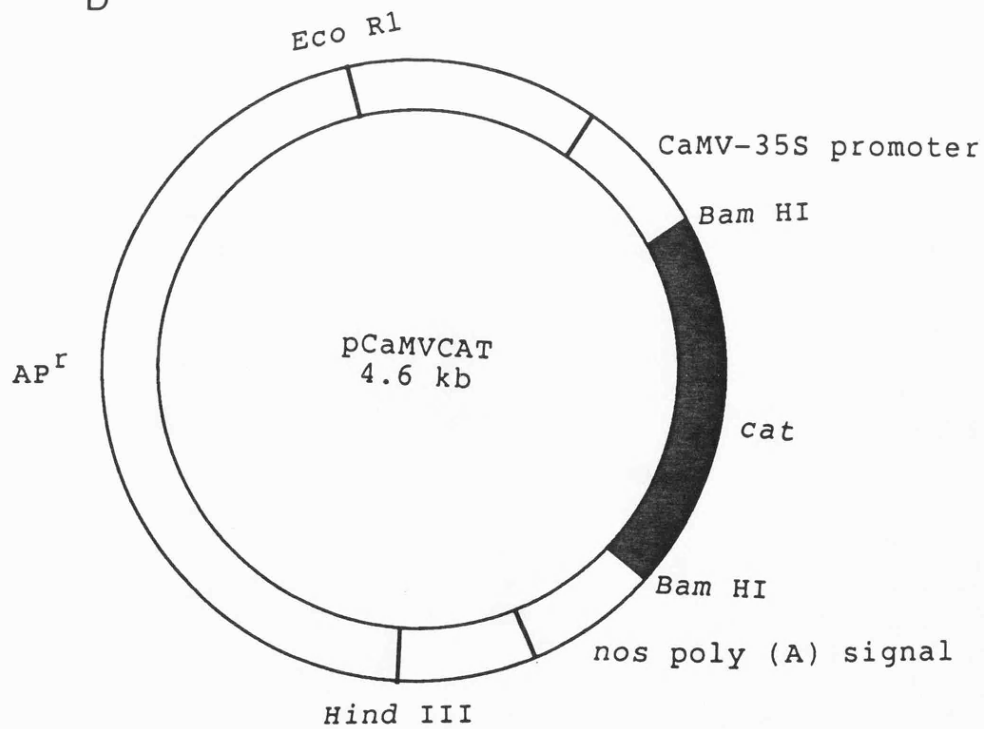
- (A) pBI.121
- (B) pBI.221
- (C) pCaMVNEO
- (D) pCaMVCAT



C



D



incubated for 4 min on ice. 60 ml of 3 M KOAc pH 5.2 was added, mixed well and centrifuged at 8000 rpm for 10 min and 4°C. The supernatant was poured off into a clean 250 ml centrifuge tube through a polyallomer wool plug held in a plastic funnel. 100 ml of propan-2-ol was added, mixed well and centrifuged at 8000 rpm and 4°C for 10 min. The pellet was gently rinsed with 70 % ethanol and redissolved in 10 ml TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 7.5). The volume was adjusted by weighing to 17.5 g by adding TE as required, then 20.79 CsCl and 3.5 ml of EtBr (5 mg/ml) solution were added, and the solution was centrifuged in a 35 ml centrifuge tube at 15000 rpm at room temperature. Beckman Ti80 tubes were completely filled and heat sealed and then centrifuged at 55000 rpm for 42 hr. The tubes were removed carefully from the rotor and examined in the dark room under UV light. At this stage bands were visible: an upper, sharp band of chromosomal DNA and a lower, diffuse band of plasmid DNA. The top of the tube was punctured and a hypodermic syringe needle was inserted through the side of the tube below the lower band, and the lower band was drawn off in as small a volume as possible and transferred to a sterile Universal bottle. The EtBr was completely removed by partitioning the upper (aqueous) phase several times with an equal volume of propan-2-ol saturated with CsCl and H₂O. The plasmid was precipitated by adding 0.1 volume of 2 M NaOAc pH 5.6 and 3 volumes of ethanol followed by mixing and incubation in a dry ice/bath for 15 min. The mixture was centrifuged at 10000 rpm and 4°C for 15 min, then the supernatant decanted and the pellet was rinsed with 70% ethanol and dried in a

vacuum desiccator. The plasmid was redissolved in 300 μ l of Q water. The DNA concentration was determined from OD²⁶⁰ of a diluted aliquot of the plasmid solution. (Draper et al., 1988)

2.13 RESTRICTION ENZYME DIGESTION OF PLASMID DNA

The restriction digests were performed essentially according to various manufactures instructions. 1 μ l of the appropriate restriction enzyme (Eco R1 or Hind III; BRL) and 4 μ l of restriction enzyme buffer (Reaction 2 for Eco R1, reaction 3 for Hind III or reaction 4 for both: BRL) were added to 2 μ l of plasmid DNA (250 μ l/ml) and the volume was made up to 20 μ l with distilled water. The reaction was incubated at 37°C for 2 hr.

3 μ l of gel loading buffer (50% glycerol, 0.25M EDTA, 0.01 bromophenol blue pH 8.0) was added to the reaction and mixed well prior to loading on the gel.

2.14 FRACTIONATION OF PLASMID DNA BY AGAROSE GEL ELECTROPHORESIS

A gel is a complex net work of polymeric molecules. DNA molecules are negatively charged and under an electric field DNA molecules migrate through the gel at rate dependent upon their size. 0.9 g of agarose (Seakem) was added to 100 ml 1x TAE buffer (89.0 mM boric acid, 2.5 mM EDTA, pH 8.3), dissolved completely by boiling and then cooled to 50°C. A clean Perspex plate was prepared by sealing both ends with tape and the well former was positioned into the appropriate slots. The cooled molten agarose was poured into the plate and allowed to solidify.

When the agarose had set, the slot formers and the sealing tapes were carefully removed. The gel was submerged into the electrophoresis tank and the powerpack was connected.

The digested plasmid DNA samples, marker DNA, uncut plasmid DNA were loaded into the wells avoiding the outermost lanes. 0.5 μ l/ml ethidium bromide was added to the tank to stain the bands of DNA. The gel was then electrophoresed at 90 V for 4 hr until the bromophenol blue had migrated to the bottom of the gel. DNA was viewed with a U.V. transilluminator and photographed using a Polaroid Land Camera fitted with an orange filter.

2.15 PEG TREATMENT OF TOBACCO POLLEN

Pollen from freshly dehisced anthers of tobacco was released in 4 ml of SM in a Universal bottle and then distributed into 4 bottles each containing 1 ml of medium. Various concentrations of PEG 6000 (0%, 10%, 15%, 20% and 25% w/v) were added, then the tubes were spun down for 1 min at 6500 rpm. The supernatant was removed with a Pasteur pipette leaving a thin film of medium on top of the pellet. 20 μ l of plasmid solution CaMVNEO (300 μ g/ml) was added to each pellet. The pellet was resuspended gently using a Pasteur pipette and left at room temperature for 1 min. 2 ml of an appropriate PEG solution was added by running down the side of the tube and the germinated pollen was resuspended by rolling the tube between hands until a fine suspension was achieved, then incubated at 25°C for 90 min with occasional mixing. The incubation mixture was diluted stepwise by slowly adding 10 ml of SM (2 ml every 3 min), The germinated

pollen was then spun down for 1 min, the supernatant removed (as described earlier) and the germinated pollen applied to 15 stigmata in emasculated flowers.

2.16 ELECTROPORATION OF POLLEN TUBES WITH 6-CARBOXYFLUORESCEIN

Tobacco pollen was germinated in 1 ml SM in the presence of the dye 6-CF; 200 μ l of the dye (10 mg/ml 0.1 M NaOH) was added to 1 ml of SM media. The germinated pollen was subjected to electroporation with a pulse duration of 10 μ s: 50v, 100v and 150v using a Kruss AT 750 electroporator. The pollen was then placed in 2 ml of SM medium and incubated for an hour at 25°C. The germinated pollen solution was then pelleted by centrifugation for 25 sec at 6500 rpm. The supernatant was discarded and the germinated pollen was resuspended in the 6-CF, left at room temperature for 5 min and then viewed under the U.V. microscope

2.17 BIOTIN-LABELLING OF DNA TO DEMONSTRATE ITS UPTAKE AND POSITION IN GERMINATING PEA POLLEN

2.17.1 Labelling the plasmid with biotin

10 μ l of plasmid CaMVNEO (1 μ g/ μ l) was mixed with 10 μ l biotin (photobiotin acetate) in an Eppendorf tube. The mixture was placed in ice at a distance of 12 cm below a 500 Watt Mercury vapour lamp (Tungsten lamps are not recommended because of their high heat output) under 1 cm water below the lamp for 20 - 30 min. 10 μ l of 0.1 M Tris-HCl pH 8 and 70 μ l of Q water were added to the mixture to give a final volume of 100 μ l. The labelled

plasmid was then extracted (twice) with 100 μ l 2-butanol. The extraction was done by mixing the two liquids, spinning the tube for 1 min, then removing the upper layer. 30 μ l was left which contained the labelled plasmid. 7.5 μ l 3 M NaOAc and 100 μ l ethanol were added, mixed by whirlmixer and the DNA precipitated in a dry ice/ethanol bath for 1 hr, and spun for 20 min. The supernatant was discarded and the pellet was washed in 70% ethanol and dried under vacuum (if a pellet is visible it should be red in colour). The pellet was dissolved in 50 μ l Q water without EDTA because it inhibits pollen germination.

2.17.2 Visualisation of the labelled plasmid.

40 μ l of SAAP (Streptavidin alkaline phosphatase) was added to each slide over the pollen, then the slides were incubated for 2 hr at room temperature. The slides were washed twice in 1 ml of AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂). 40 μ l of alkaline phosphatase substrate was placed onto each slide and incubated for a minimum 4 hr or overnight. If the colour development appeared sufficient the slides were washed with AP STOP (20 mM Tris-HCl pH 8, 5 mM EDTA), they were then examined under the microscope (x10 and x60 under oil).

2.18 RADIOLABELLING PLASMID TO DEMONSTRATE DNA UPTAKE INTO TOBACCO POLLEN TUBES

2.18.1 Fractioning the plasmid and radiolabelling

Plasmid DNA CaMVNEO was digested with restriction

enzyme *Hind* III resulting in two fragments (2.72 kb and 1.68 kb). The fragments were end-labelled with ^{35}S radioactive ATP (Draper et al., 1988) by combining the following into a sterile Eppendorf tube:

3 μl (500 ng) DNA

3 μl (0.5 mM) d CTP \neg

3 μl (0.5 mM) d TTP | (Pharmacia cat. no. 27-2166-01)

3 μl (0.5 mM) d GTP \neg

3 μl ^{35}S d ATP (Amersham cat. no. SJ 304)

3 μl DNA polymerase I 'Klenow fragment': 1 unit/ μl
(Pharmacia cat. no. 27-0928-01).

12 μl Q water

The total amount of 30 μl was incubated for 2 hr. The reaction was stopped by adding 70 μl of stop solution (20 mM NaCl, 20 mM Tris-HCl pH 7.4, 2 mM EDTA, 0.25% SDS). 1 μl of the total volume was spotted in the centre of a Whatman GF/C glass-fibre disk (2.4 cm) and air dried. Another 1 μl of the reaction mixture was aliquoted into an Eppendorf tube containing 500 μl of a solution of herring sperm DNA (500 $\mu\text{l}/\text{mg}$ in 20 mM EDTA). The mixture was precipitated with 125 μl of ice-cold 50% TCA (Trichloroacetic acid) and chilled on ice for 5 min. The precipitate was collected by filtering the solution through a GF/C disk in a filter disk and washed twice with 5 ml ice-cold 10 % TCA, followed by 2x5 ml of 100% ethanol. The filters were dried and placed in a plastic scintillation vial.

Both scintillation vials were filled up with 5 ml of a toluene based scintillation liquid and the activity counted using an LKB 'Beta Rack' liquid scintillation

counter. The unwashed filter gave the total radioactivity in the sample and the washed filter gave the incorporated counts. Around 1% was the incorporated counts in most reactions.

2.18.2 Autoradiography and staining

In a totally dark room lacking safe light or fluorescent bulbs (because they glow even when off) and also without rubber gloves (because of the static sparks), 10 g of emulsion was added to 6 ml of water/glycerol (1 ml of glycerol was dissolved in 59 ml of Q water and autoclaved), and mixed. The emulsion was left to melt in a waterbath at 45°C for 1 hr, during this period the emulsion (Nuclear emulsion, K2, ILFORD) was mixed by gentle inversion at least once. The emulsion was mixed again then poured into dipping chamber to within 3-4 mm of the top. Each slide was dipped into the dipping chamber, holding it by the frosted end (the dipping chamber was refilled as necessary). The emulsion was carefully wiped from the back of the slide, and the slides stood on end at about 30° to the vertical in a Perspex rack. The slides were drained and dried in this position at room temperature for at least 2 hr or overnight. The slides were transferred to a light-tight slide box with silica-gel, sealed with plastic tape and stored at 4°C away from sources of radiation and strong electromagnetic fields.

2.18.3 Development

The slides were removed from fridge and unwrapped

under a safe light or in a total darkness and immersed in D19 (Kodak D19, 160 g was dissolved in 1 litre of Q water. Cat No. 502 7005) for 2 - 4 min, then immersed in 1% acetic acid, followed by immersing in 'Unifix' (Kodak, Cat. No. 501 10510; 1 litre of Q water was heated to 65°C and 150 g 'Unifix' powder was added and stirred constantly until dissolved), for 4 min, after 2 min the light could be switched on. The slides were then immersed in water for 2 min then left under running tap water at room temperature for at least 30 min.

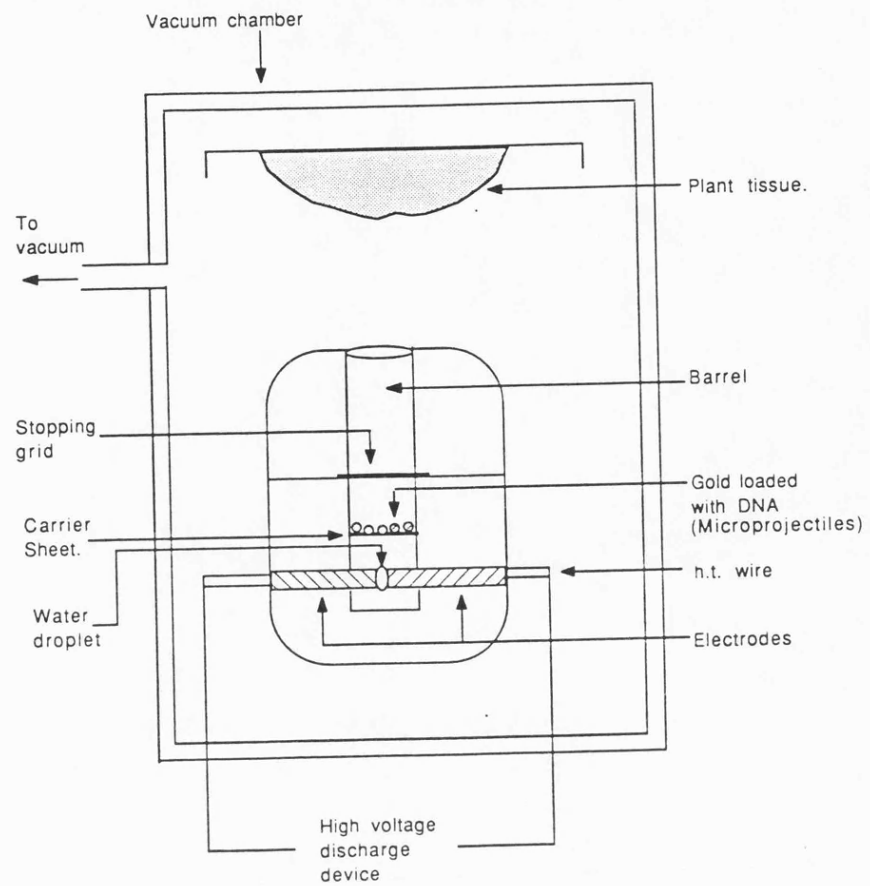
2.19 MICROPROJECTILE MEDIATED TRANSFORMATION OF TOBACCO AND PEA POLLEN

The microprojectile unit (MPU) consist of a Teflon cylinder with a hole drilled in it - rather like a gun. Two brass electrodes are at the bottom of the barrel. When a high tension current is passed across these electrodes a spark is produced (Fig. 2.2 A). A carrier sheet which has been loaded with gold particles coated with DNA is placed above the electrodes, and above this sheet is a stopping grid. Water is placed between the electrodes which vaporises explosively when the high tension current is passed across. This explosion accelerates the carrier sheet towards the stopping grid. The sheet is stopped by the grid, but due to inertia, the gold particles carry on to move through the stopping grid, forcing their way into the plant tissue, delivering their DNA to transform the target cells (Fig 2.2 B).

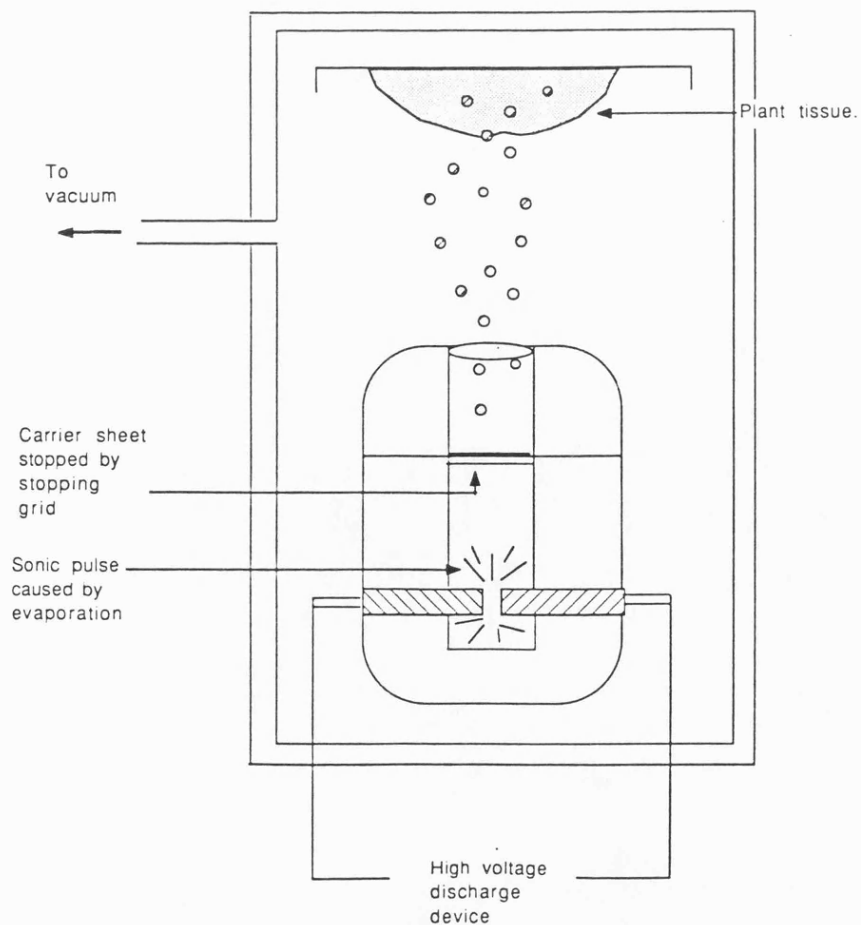
Fig. 2.2 Structure and operation of microprojectile gun
(designed by Mr N. Griffiths, Botany Department,
Leicester University).

- (A) Before firing
- (B) After firing

A



B



2.19.1 Preparation of mylar sheets

Carrier sheets were punched out using 11 mm punch. They were sterilised by autoclaving for 20 min at 121°C. The sheets were rinsed in 1% solution of HEC (Hydroxyethyl-cellulose), and left to dry in a Petri dish with the lid ajar.

2.19.2 Loading DNA onto gold particles

10 mg of particles were autoclaved as mentioned above (section 2.19.1), then rinsed in a 0.02% solution of poly-L-lysine and left to dry. 20 μ l of plasmid pBI. 221 (300 μ g/ml; Fig. 2.3) was added to 80 ml of Q water, then they were mixed and left for 10 min. 10 μ l of 10 mM Na_2HPO_4 and 10 mM of CaCl_2 were added to the mixture, then left for 10 min to precipitate DNA onto the gold particles. The supernatant was removed, then vacuum infiltrated at 65°C for 5 - 10 min. The pellet was then resuspended in 100% ethanol. The particles were loaded onto the mylar sheet (see the next section).

2.19.3 Loading of sheets

25 μ l of the ethanol solution was placed onto the prepared sheet and allowed to air dry until all the ethanol was dried off, giving an even spread over the surface of the sheet.

2.19.4 Operation of the microprojectile unit (MPU)

Before use, the Ignitron (high voltage switch) has to be electronically 'aged'. The light and the fan must be

turned on at least two hr before the system is used. This creates a heat gradient, ensuring that the mercury does not condense onto the top (anode) of the Ignitron.

The particle gun (PG) was disassembled. 5 - 7 μ l of sterilised tap water was loaded between the discharge pins of the PG. The prepared mylar sheet was placed onto the slot of the retaining plate. The PG was reassembled, taking care not to lose sterility. The PG was placed into the vacuum chamber and the particle hole was positioned directly below the sample. The cover was put onto the vacuum chamber and the vacuum inlet valve was closed. The vacuum chamber was evacuated and the PG was connected to the MPU. The safety short-out was raised and the power supply was turned on to the high voltage supply and trigger circuits. The trigger was switched on so the green light came on. This means that the trigger capacitor was being charged. In this step, the voltage knobs on the power back were turned down, then the high tension switch was pushed and the voltage was raised to the desired level. When the ampage has reached zero, the capacitors were fully charged (the system is ready to be fired). The high tension was turned off and the trigger was switched so that the red light came on (the trigger is in firing mode). For firing, the trigger button was pushed. The main supply to vacuum pump (trigger and power pack) was turned off and the safety plate was dropped off and the red plugs were unplugged. The vacuum was slowly released then the PG was removed from the chamber.

2.19.5 Bombardment of tobacco and pea pollen

Freshly dehisced anthers were crushed and mixed in 1 ml of SM or BM in an Eppendorf tube. 100 μ l of the stock was added to 900 μ l of fresh media and left to germinate for 0, 15, 30, 45, 60, 90 min. The pollen for each treatment was centrifuged for 30 sec, then the supernatant was removed leaving 150 μ l of concentrated pollen which was placed onto three sheets (50 μ l on each) for every treatment. Both pea and tobacco pollen was bombarded. Pollen at early developmental stages (tetrad stage) was also bombarded. Pollen as a control was left without bombardment. After 16 - 24 hr, the histochemical assay was performed for the treated pollen and the controls.

2.20 FUSION OF THE TOBACCO POLLEN TUBES WITH LIPOSOMES

2.20.1 Source and storage of lipids

- Cholesterol was obtained from Sigma (m.w 386.669), and was stored in -20°C . A stock solution of 19.3 mg in 10 ml of chloroform (50 $\mu\text{M}/\text{ml}$) was used and at the required time the stock was diluted x10 to give 5 $\mu\text{M}/\text{ml}$.

- Phosphatidylserine was purchased from Lipid Products Ltd., Nutfield, Surrey, in sealed ampoules which were stored at -20°C . 100 mg was dissolved in 5 ml chloroform:methanol giving a colourless solution.

- Lecithin mixture was purchased from Lipid Products Ltd., Nutfield, Surrey; supplied in chloroform:methanol solution in 1 ml. Ampoule containing: 42 μM Egg lecithin. 12 μM cholesterol. 6 μM stearylamine.

2.20.2 Preparation and purification of 6-carboxy-fluorescein (6-CF) and other dyes.

Fluorescein (6-CF) was purchased from Sigma (No. C-0662, m.w. 376.3), stored at 0 - 5°C. 0.07 g was dissolved in 2.5 ml SM (75 mM). A cloudy mixture was obtained, then was cleared by filtering through 0.45 μ m filter (Acrodisk. No 6224184. Gelman Sciences. U.K.). After dissolving 6-CF, the media became acidic and so 0.1 M NaOH was added till the pH became 6.5.

Fast green (1% fast green in I.M.S.) and acetocarmine (2% carmine in 45% acetic acid) were used to colour the liposomes. 20 μ l was dissolved in 1 ml media. The stain was filtered through 45 μ m before use. They showed the best result under phase contrast. Quinocrine (1%; one gramme dissolves in about 35 ml water), showed the best result under U.V. giving a blue excitation.

2.20.3 Pollen germination media

Pollen germination media must be osmotically the same as liposome buffer to prevent bursting. Pollen was germinated in a variety of media. SM (Tobacco media) pH 6.5 as a control, the pollen also was germinated in TES buffer (2 mM TES pH 7.4, 2 mM L-histidine, 150 mM NaCl 0.1 mM EDTA) and in a 50% SM and 50% TES buffer.

2.20.4 Preparation of reverse phase evaporation vesicles

Liposomes were prepared according to Szoka and Papahadjopoulos (1978) as modified for the encapsulation of SV 40 DNA (Fraley, et al., 1981). All glassware was

washed with hot water and Decon, then rinsed with IMS or acetone. Throughout the procedure the temperature was maintained at 34°C in water bath and after switching on the waterbath, it was allowed one hour to stabilise.

210 μ l of phosphatidyl serine and 500 μ l (5 μ M) of cholesterol were mixed very well in a screw-cap test tube (13x100 mm, Corning Ltd., with a 'Teflon' liner). The cap was removed and the tube connected to a rotary evaporator and the solvent was removed under reduced pressure. The lipid was redissolved in 0.5 ml ether, and 0.16 ml of a sterile buffer (2 mM TES pH 7.4, 2 mM L-histidine, 150 mM NaCl, 0.1 mM EDTA) containing substances to be encapsulated (e.g. DNA or dye). Two layers were formed; the top layer was the buffer and the bottom layer was the lipid. An emulsion was made by sonicating in bath-type sonicator (Type KB 80/3; Kerry Ultrasonic Ltd., Basildon, UK) for 30 sec. The tube containing the one phase dispersion was purged with nitrogen and replaced in the rotary evaporator. Ether was removed under vacuum (300 mm of Hg) whilst rotating at 50 rpm at a temperature of 34°C, until a semisolid lipid gel was formed. The pressure was then reduced to 650 mm of Hg and left for about 1 hr to give the liposome suspension. The liposome suspension was made by adding 100 μ l encapsulation buffer and then stored under N₂ at 4°C.

2.20.5 Separation of liposomes on Ficoll gradient

The liposomes in suspension could be separated from unencapsulated materials by flotation on a discontinuous Ficoll gradient as described by Fraley et al., (1980). A

fair proportion of liposomes were lost in the procedure. So, it was only necessary where liposomes containing dyes such as 6-CF needed to be visualised (the presence of unencapsulated florescent dye cause background fluorescence which makes the liposomes difficult to observe under the U.V. microscope). 1 ml of liposomes suspension, 1 ml of 30% Ficoll, 1 ml of 10% Ficoll, 1 ml of 5% Ficoll and 1 ml of encapsulation buffer were gently layered consecutively in a 5 ml plastic centrifuge tube (it was recommended to lay the solutions by placing the pipette at the bottom of the tube for each layer). The tube was then centrifuged at 3000 rpm in SWTi 65 rotor in a Beckman ultracentrifuge for 30 min at 20°C. The liposomes banded just below the layer of buffer which was removed with glass pipette, whilst the encapsulated materials (e.g. 6-CF or DNA) remained in the most dense Ficoll layer.

2.20.6 Uniformity of the liposomes

A more uniform suspension of liposomes were obtained by passing the diluted suspension through 0.45 μ m filter, this broke the aggregated liposomes and removed large particles of lipid.

2.20.7 Monitoring plasmid encapsulation and protection from DNase

120 μ l (20 μ g/ml) plasmid (pCaMVNEO) was encapsulated in liposomes. The liposomes were then washed in liposome buffer (see Section 2.19.5), then burst by addition of 10 μ l of Triton X-100 to release the plasmid.

In another treatment, DNase was added to the plasmid containing liposome. Phage lambda DNA was used as a standard. The DNA samples were then run in an agarose gel (see Section 2.14) for 3 hr at 90 V. The extracted plasmid appeared on the gel as an intact band (Fig. 2.7; lane 1), showing that the liposomes contain plasmids and that thus had not been degraded. Naked Plasmid which was treated with DNase (2 μ l DNase/0.5 μ g of DNA) was completely degraded (Fig. 2.7; lane 3).

2.20.8 DNase treatment, washing, and staining of pollen tubes

In order to show the effect of DNase inside/outside the pollen tube. Pollen tubes were incubated with herring sperm DNA in BM. DNase (2 μ l DNase/0.5 μ g of DNA) was added, left for 20 min, flicking the tube every 5 min, then 3 washes were made by centrifuging the tube for 10 sec, discarding supernatant and addition of fresh media. The tube was then left for a further 1 hr and 20 μ l quinacrine was added to stain the herring sperm DNA.

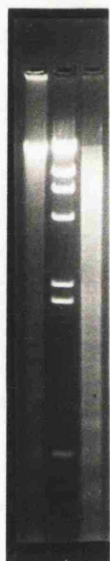
2.21 WHEAT SEEDLING SELECTION ON CHLORAMPHENICOL AND KANAMYCIN

As the wheat pollen was difficult to germinate, an experiment was designed to introduced naked plasmid into wheat ovules in vivo (see Chapter 5; section 5.6). The wheat seeds were tested for selection on chloramphenicol and kanamycin.

20 wheat seeds were placed on a 1 cm layer of fine polystyrene beads, which was placed on 25 ml water in

Fig. 2.3 Liposome-encapsulated plasmid was released from DNase-treated liposomes and appeared as an intact band (lane 1), lambda DNA, digested with *Hind* III and *Eco* RI (lane 2), and plasmid DNA degraded in presence of DNase (lane 3).

1 2 3



beaker containing different concentrations of chloramphenicol (0, 10, 25, 50, 100, 150, 300, 500 $\mu\text{g/ml}$), or kanamycin (0, 50, 100, 150, 200 $\mu\text{g/ml}$), and germination monitored.

CHAPTER **3**

3.0 IN VITRO POLLEN GERMINATION AND SUBSEQUENT FERTILISATION OF TOBACCO AND PEA

3.1 GENERAL INTRODUCTION

In vitro pollen germination is an important technique used in many areas of plant science. Its usefulness in the study of pollen tube physiology and incompatibility is without doubt.

Pollen tube cytology and physiology may differ under artificial and natural conditions. *In vivo*, the pollen tubes are closely associated with the tissues of the stigma and style and the growth is dependent on their complex metabolic products. This association makes it almost impossible to follow the normal growth of pollen tubes *in vivo* and to study their metabolic requirements and growth characteristics. The technique of pollen

culture *in vitro*, therefore, allows one to germinate and study the growth of pollen in chemically defined media, under strictly controlled experimental conditions (Vasil, 1987).

The ability of pollen to germinate and grow in culture varies greatly between species and largely depends upon whether they contain two cells (bicellular) or three (tricellular) at anthesis; the former generally germinate well in culture and can be stored successfully for long periods of time, whereas the latter usually do not germinate, or else do so poorly and do not store well (Vasil, 1987).

In this present study the aim is to establish an *in vitro* germination system and an effective fertilisation system with *in vitro* germinated pollen. High percentage germination of pollen tubes (>95%) is required and the growth should be rapid and fairly synchronised and have no (or a short) lag phase before pollen germination. The pollen tubes should be long, robust and morphologically normal. They must be germinated at high densities and large quantities of pollen should be easily handled especially during pollination without damage such as bursting. The objective of the fertilisation system is to be able to effect reasonable levels of seed set and to produce viable seeds. It is then hoped that growing pollen tubes could be targets for vector DNA uptake and fertilisation could be a means of introducing foreign genes into plants.

Generally the mature pollen or previously germinated pollen should be placed on the stigma and will

germinate into the style; the male gametes should then be taken to the ovule. Thus, if vector DNA can be carried down with the 'male germ unit' then there is a chance that transformation will ensue.

The fertilisation biology of many plants has been studied out *in vivo* and *in vitro* with the purpose of overcoming sexual incompatibility (for reviews see section 3.9).

Recently, interest has arisen concerning the use of pollen as a means of introducing foreign genes into commercially important crop species (see Chapter 4; section 4.1.1) and in the possibility of selection being exerted on the gametophyte generation (Hodgkin, 1987; Laughnan and Gabay, 1973; Pfahler, 1983). An effective *in vitro* germination system is essential for many of the approaches suggested.

3.2 TOBACCO AND PEA REPRODUCTIVE BIOLOGY

3.2.1 Natural fertilisation system of tobacco and pea

Both pea and tobacco have been investigated with regards to fertilisation biology. The timing of dehiscence was monitored to estimate when the anthers dehisce naturally under the green-house conditions and the biology of pollination and germination were examined, to estimate when the pollen tube penetrates into the stigma and when it arrives to the ovule to deliver its vegetative cell and two sperms.

3.2.2 Tobacco

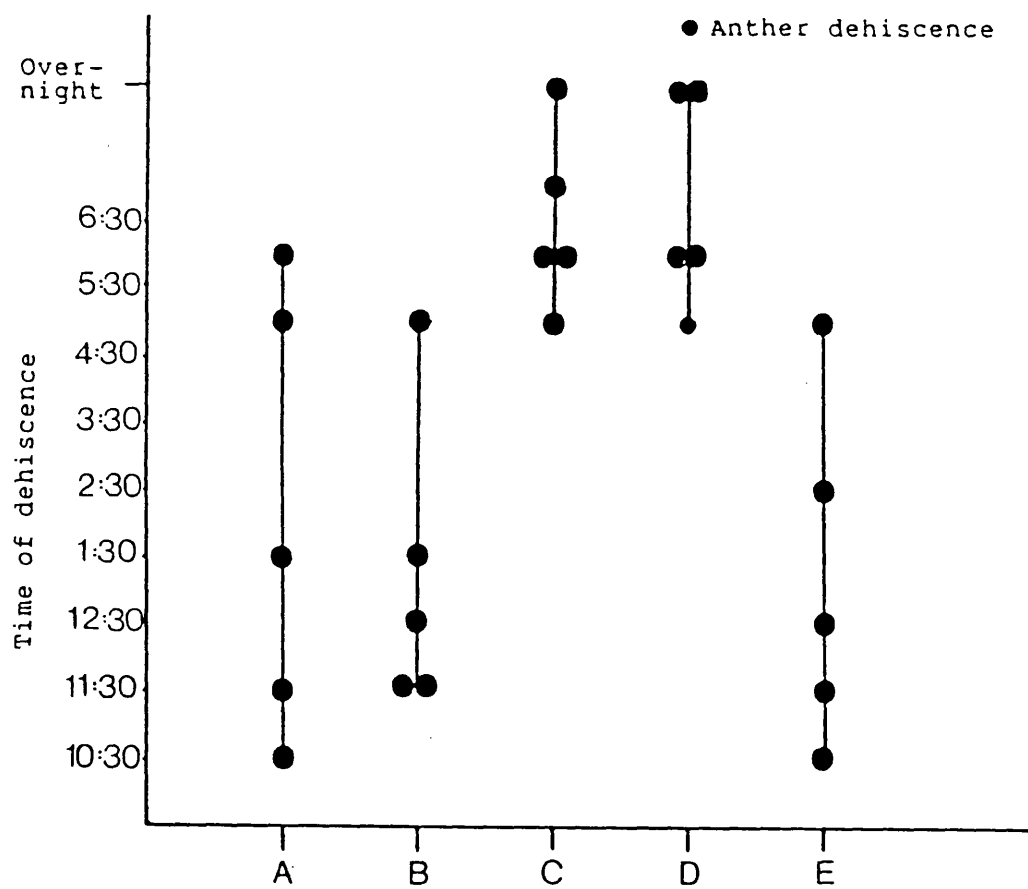
3.2.2.1 Dehiscence time

Tobacco flowers from different genotypes were labelled and kept under green-house conditions. These flowers were examined for anther dehiscence at hourly intervals for 9 hr starting at 9.00 am and again after 24 hr (3 replicates). In tobacco the time of dehiscence varied greatly between anthers of the same flowers (Fig. 3.1). For instance, the first anther to dehisce in one flower studied (A; Fig. 3.1) did so at 10.30 am (5 hr after daybreak), the second anther dehisced at 11.30 am, the third at 1.30 pm, the fourth at 5.00 pm and the fifth at 6.00 pm. Some flowers (C,D) started to dehisce in the afternoon or in the early evening and continued dehiscence overnight. Thus, in tobacco there was no specific time between the dehiscence of one anther in a flower and of another. Moreover, it was impossible to predict exactly when anthesis would occur in any one anther.

3.2.2.2 Germination biology

Pollen was dusted onto stigmata of tobacco plants grown in the green-house. Most tobacco pollen grains had germinated and penetrated the stigma after a 4 hr incubation. The stigma and the style were then fixed, squashed then stained with aniline blue (see Chapter 2; section 2.7) and further observations were made. During the first 2 hr, most pollen was observed to hydrate but there was no germination. During the third and fourth hours most pollen had germinated and penetrated into the

Fig. 3.1 Time of anther dehiscence in five flowers
of tobacco.



stigma (Fig. 3.2). When the pollen pore was in contact with the stigma, the tube tended to penetrate into the stigma directly, however, in most cases the pollen tube grew for some length before entry into the stigma surface.

Emasculated flowers were pollinated and after 8 hr and 12 hr, the styles were fixed and squashed. The tube length was 3.4 mm after 8 hr (Fig. 3.3A) and 8.3 mm after 12 hr (Fig. 3.3B). Pollen grains and pollen tubes were stained in analine blue and the generative and vegetative cells were observed (Fig. 3.4 A&B). The first callose plug was observed between 250 μm and 300 μm and the second callose plug between 550 μm and 600 μm and approximately every 300 μm , a callose plug was formed (Fig. 3.5)

3.2.2.3 Time between pollination and fertilisation

Emasculated flowers from different genotypes were pollinated, then their styles removed after 5, 10, 15, 20, 23, 25, 27, 30, 35, or 40 hr (2 replicates). It was found that no seeds were obtained before 30 hr and this means that the tubes did not reach the ovules until at least 30 hr, post pollination.

3.2.3 Pea

3.2.3.1 Dehiscence time

Flowers in green-house grown plants from different pea genotypes (3 replicates) were examined for dehiscence time. In pea, unlike some other species there was no single time of dehiscence observed but dehiscence was not seen before 11.00 am (i.e. 6 hr after sunrise).

There was almost as much variation in the time of

Fig. 3.2 Germination and penetration of tobacco pollen tubes into the stigma, stained with aniline blue 6 hr after applying onto the stigma (x 81).

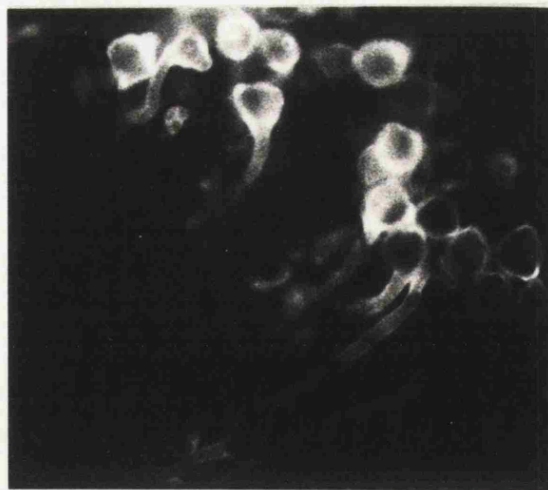
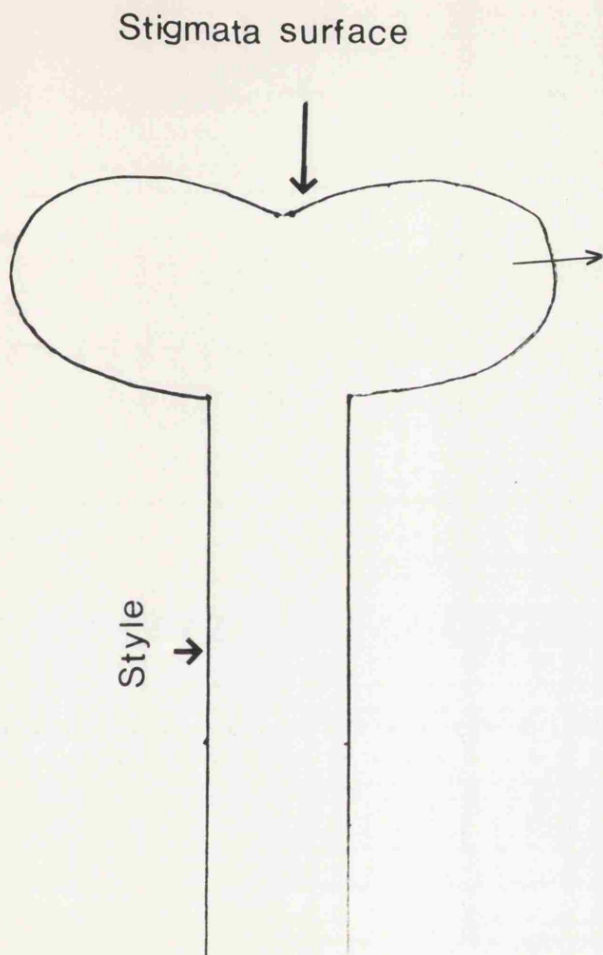


Fig 3.2

Fig. 3.3 Tube length of tobacco pollen *in vivo* after 8 hr (A; x 275), and after 12 hr (B; x 68), stained with DAPI.

Fig. 3.4 The vegetative and generative cell in tobacco pollen grain (B; x 950), and in pollen tube (B; x 950), stained with DAPI.

Fig. 3.5 Callose plugs in tobacco pollen tube after 3 hr incubation (x 95).

Fig 3.3 A



B

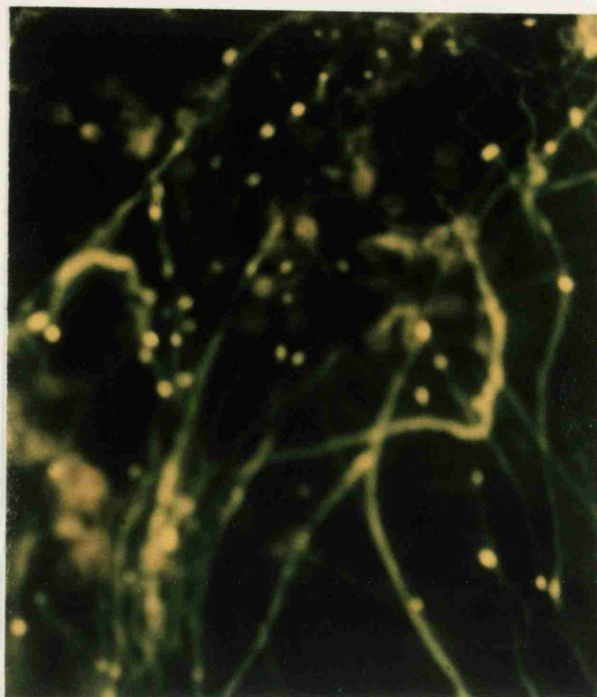
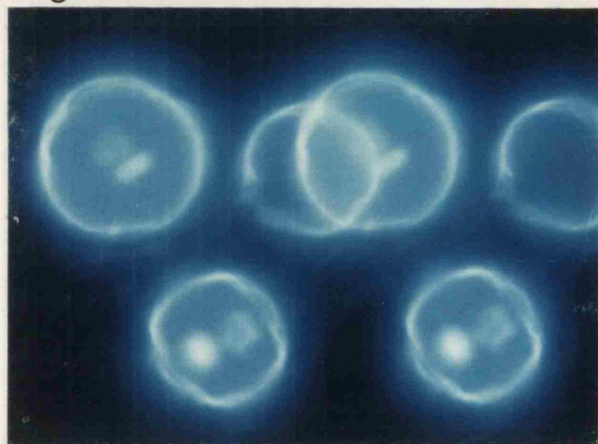


Fig 3.4 A



B



Fig 3.5



dehiscence between anthers of a single flower as there was between different genotypes (Fig. 3.6). Generally, for any flower, anthesis started between 11.30 am and 2.30 pm and lasted for at least 3.5 hr, typically exceeding 5 hr. No regular pattern of anthesis was observed between different anthers of the same flower.

The wide time spread in anthesis for anthers of a particular flower dispels the assumption that all anthers of a single flower are of the same physiological state. Furthermore, the absence of a sharp anthesis period makes the bulk collection of pollen from freshly dehisced anthers problematical. However, the absence of any anthesis prior to 11.00 am does allow emasculation on the same day that dehiscence would ordinarily occur.

3.2.3.2 Germination biology

In pea, the style is relatively long (0.5 - 1.0 cm) and covered in long hairs on the adaxial surface. In contrast, the stigma is a small structure, situated at the distal end of the style (Fig. 3.7).

On the day of dehiscence, the anthers grow up inside the keel towards the stigma, dehisce and deposit pollen all over the top of the style and the stigma. Although thousands of pollen grains are released onto this region, only 1 - 20 of those which land onto the stigma actually germinate and penetrate (Fig. 3.8). Very few of those which land on the style germinate and these do not penetrate the stigma.

From this data it is clear that most of the pollen applied to an emasculated flower would not grow down the

Fig. 3.6 Time of anther dehiscence in five flowers
of pea.

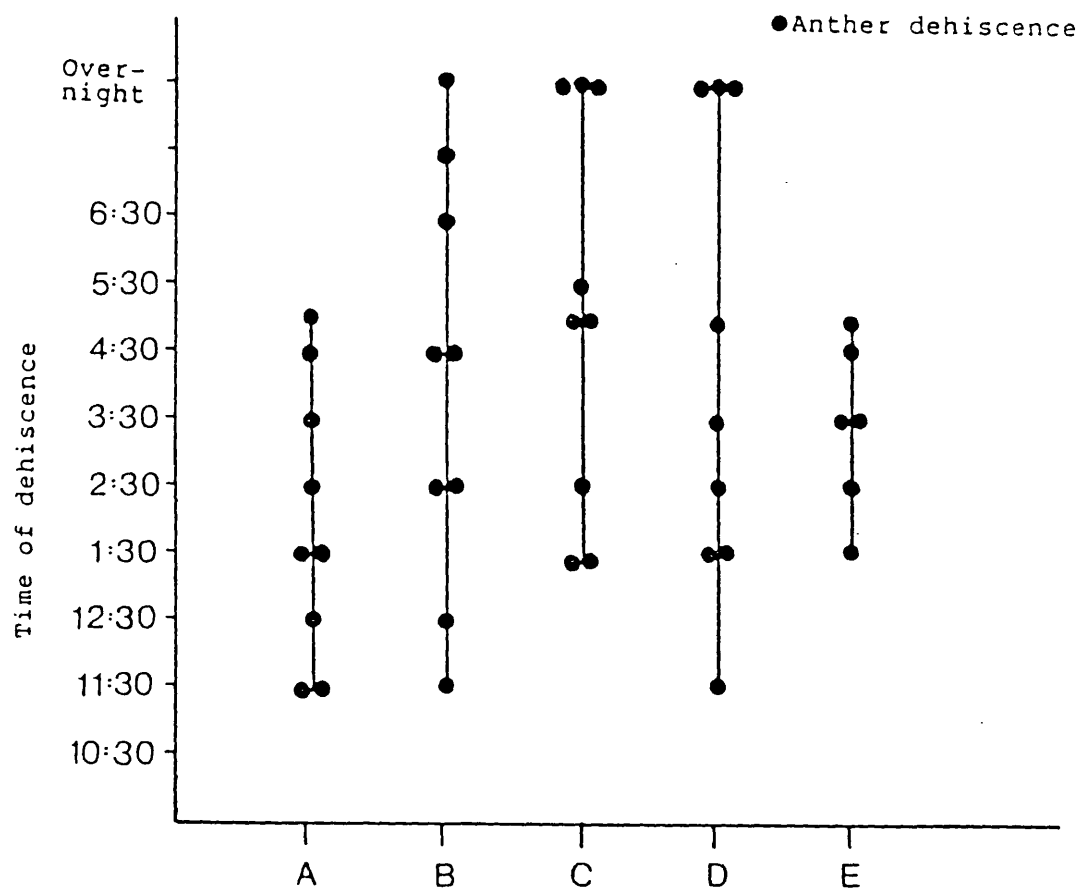


Fig. 3.7 Pea stigma is a small structure, situated at the distal end of the style (x 81).

Fig. 3.8 Germination and penetration of pea pollen tube into the stigma, after 5 hr in vivo (x 81).

Fig 3.8

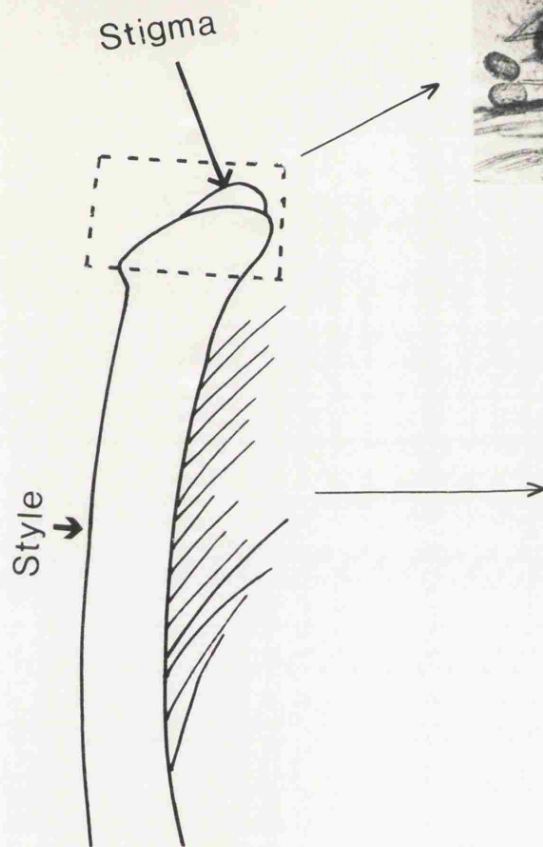
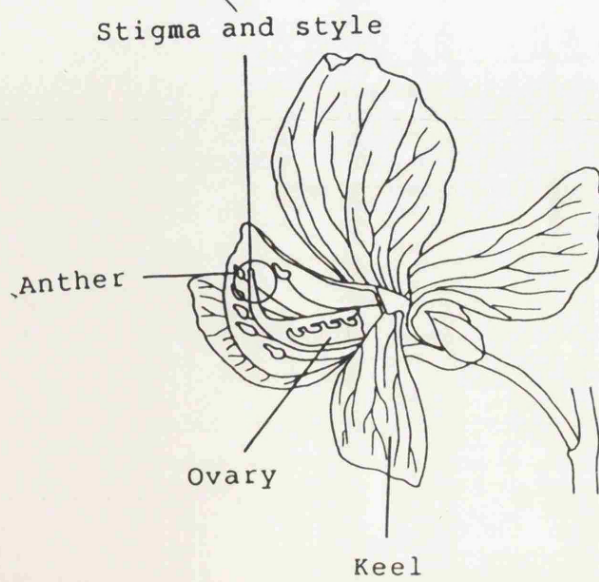


Fig 3.7



style. Thus any pollen treated with plasmid DNA should be applied only to the receptive region of the stigma.

3.3 IN VITRO GERMINATION SYSTEM OF TOBACCO

The most important factor in in vitro pollen germination is possibly sucrose and its effect on osmotic regulation and nutrition. There has been agreement in the literature over optimum conditions for the germination of tobacco and 10% sucrose was used by many groups (Tupy et al, 1955; O'Kelly, 1955; Heslop-Harrison et al., 1985).

The stimulatory effect of boron on the performance of pollen in culture was first described by Schmuker (1935) on *Nymphaea*. Tupy, O'Kelly and Heslop-Harrison suggested that 10% was the best concentration.

Tupy and Řihová (1984) reported the highest yield of tobacco pollen tubes was obtained at pH 5.9.

Heslop-Harrison reported maximum tube growth for tobacco pollen at 24°C.

3.4 RESULTS

3.4.1 The effect of media

Pollen from 5 freshly dehisced anthers from 2 flowers of one genotype was germinated in 5 different media, (3 replicates; see Chapter 2; section 2.3). There was no significant difference between tube pollen growth in SM, PM or BM (See Appendix 1) (Fig. 3.9). There was also little effect on percentage germination in the three media (Fig. 3.10).

Some inhibition of germination and pollen tube growth was observed in S media.

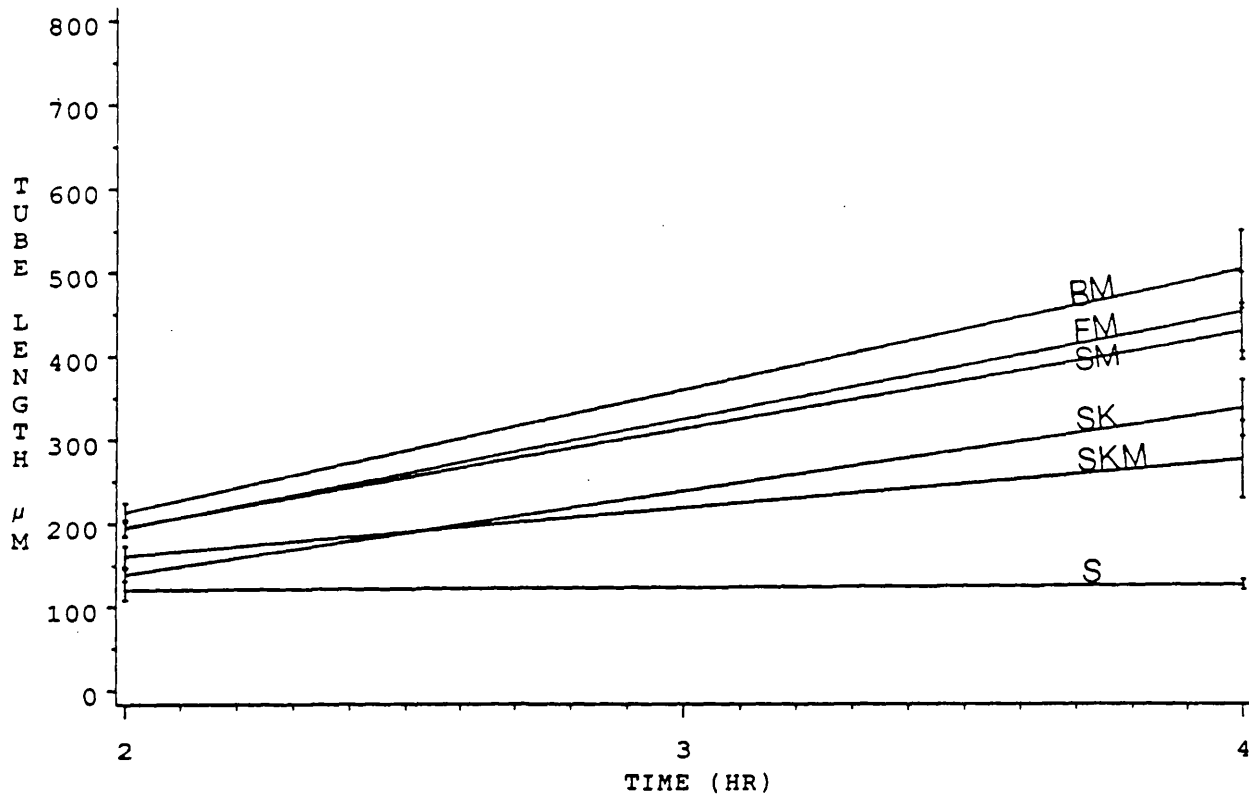


Fig. 3.9 The effect of different media on the tube growth of tobacco pollen after 4 hr (the error bars represent standard deviation).

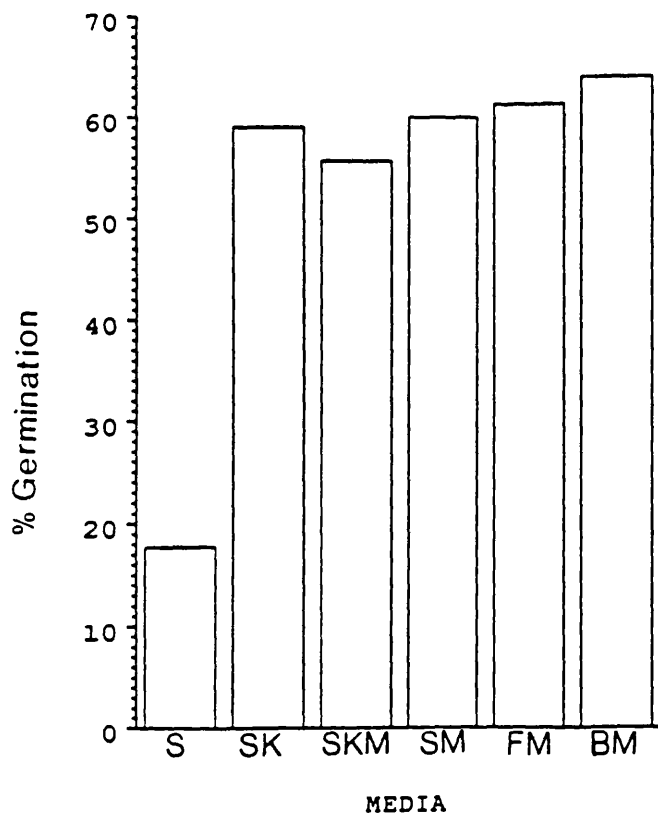


Fig. 3.10 The effect of different media on the % germination of tobacco pollen

3.4.2 The effect of concentration of sucrose

Pollen from 5 freshly dehisced anthers from 2 flowers of one genotype was germinated in 5 different media (3 replicates) containing various concentrations of sucrose and 100 mg/l boric acid. The greatest tube growth after 2 hr was in the presence of 10% sucrose, but after 4 hr and 6 hr the best growth was in 15% sucrose (Fig. 3.11 ; See Appendix 1). The percentage of germination was highest in media containing between 5% - 20% sucrose (Fig. 3.12).

3.4.3 The effect of boron

Pollen from 9 freshly dehisced anthers from 3 flowers of one genotype was germinated in media containing 15% sucrose and 0, 1, 10, 50, 100, 200, 300, 400 or 500 mg/l boric acid (3 replicates) . The highest tube growth was seen after 4 and 6 hr in media containing between 10 - 150 mg/l (Fig. 3.13; See Appendix 1). There was no tube growth in the control which contained no boric acid after 2 and 4 hr and slight tube growth after 6 hr.

Little difference was noticed in percentage of germination between 1 mg/l to 500 mg/l (84 to 93%)(Fig. 3.14).

3.4.4 The effect of pH

Pollen from 7 freshly dehisced anthers from 3 flowers of one genotype was placed into 400 μ l of SM, adjusted to PH 6.75 with 0.1 M NaOH/HCl. The solution was mixed and 50 μ l aliquots added to 1 ml samples of SM set to pH 5, 6, 6.25, 6.50, 6.75, 7 or 8. The pH was

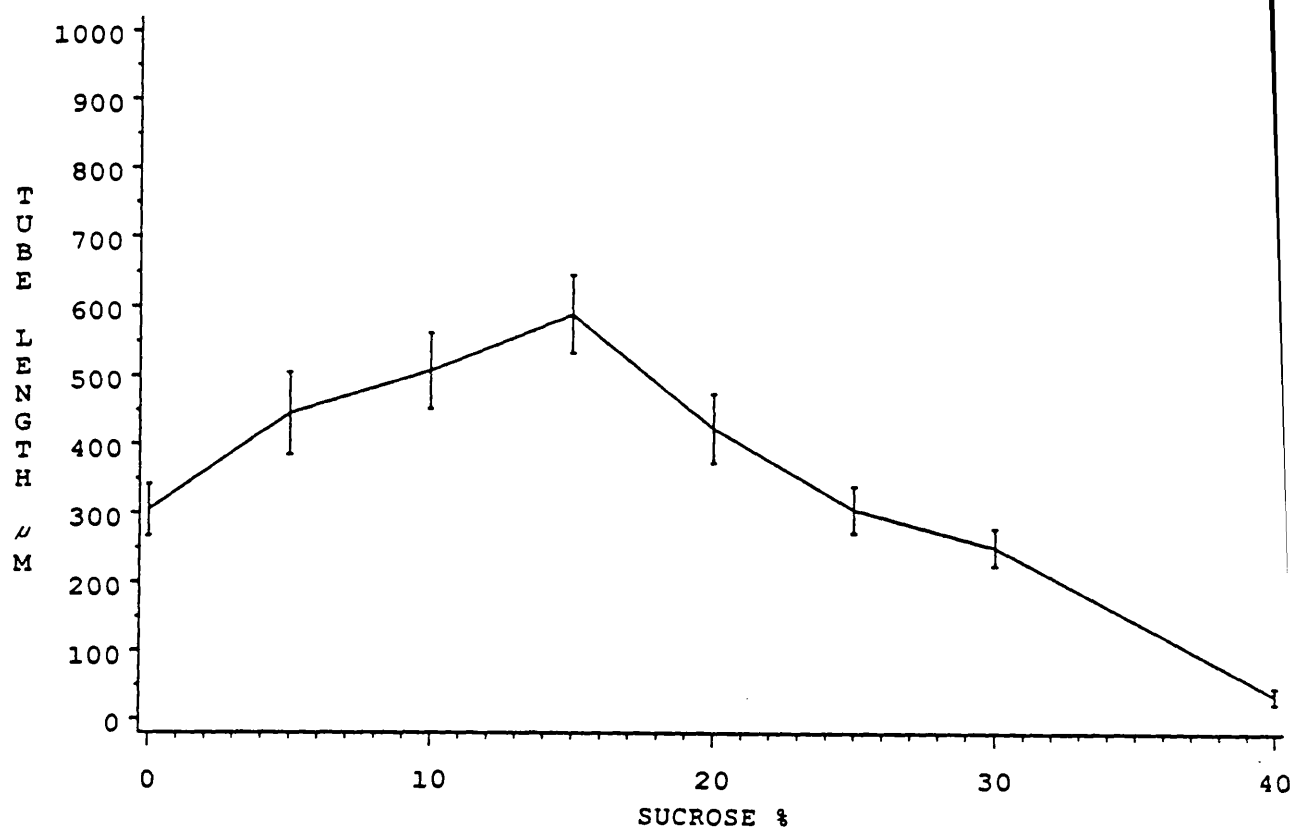


Fig. 3.11 The effect of sucrose on the tube growth of tobacco pollen after 4 hr (the error bars represent standard deviation)

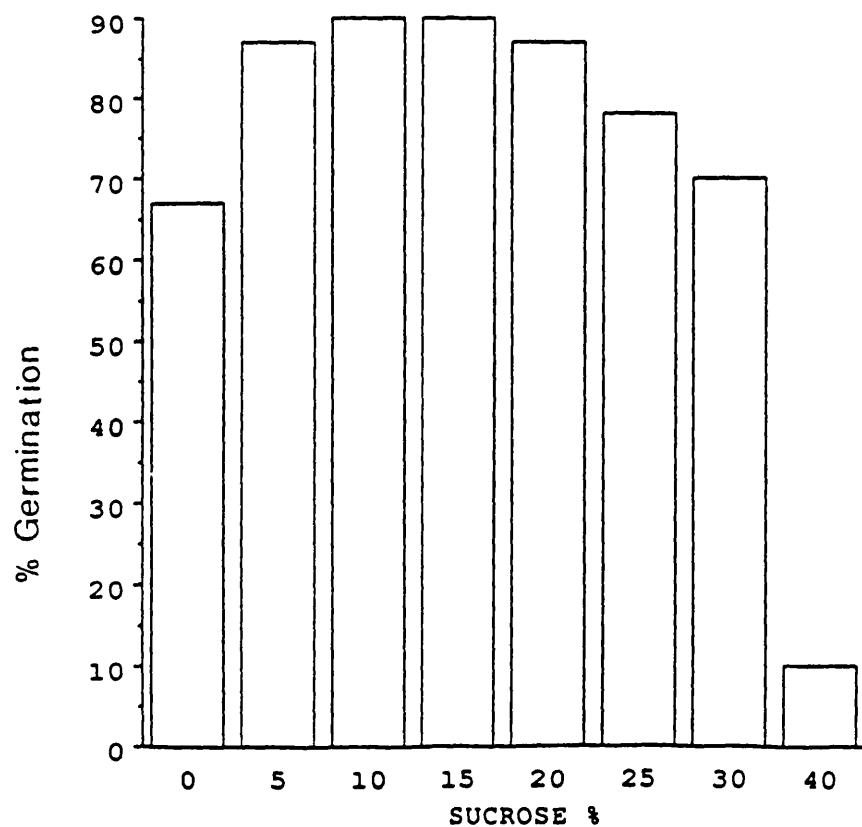


Fig. 3.12 The effect of sucrose on the % germination of tobacco pollen



Fig. 3.13 The effect of boric acid on the tube growth of tobacco pollen after 4 hr (the error bars represent standard deviation)

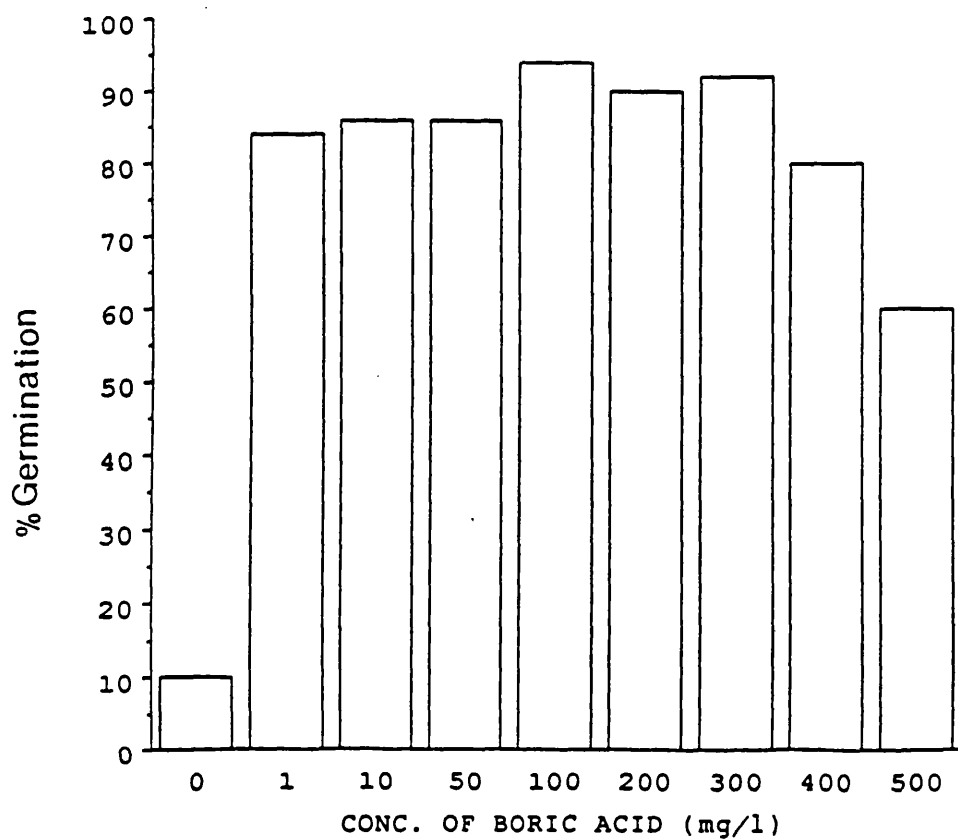


Fig. 3.14 The effect of boric acid on the % germination of tobacco pollen

readjusted to the required setting after adding the aliquots of pollen. The tube growth was greatest after 2 hr in media adjusted to pH 6 - 6.50, but after 4 and 6 hr the tube growth was greatest in media adjusted to pH 6.75 - 7 (Fig. 3.15; See Appendix 1). Some inhibition of germination was observed in media of pH 8. Overall little difference was observed in the percentage of germination at any pH level (Fig. 3.16).

3.4.5 The effect of temperature

Pollen from 7 freshly dehisced anthers from 3 flowers of one genotype was placed in 7 ml of SM adjusted to pH 6.75. The solution was mixed, divided equally into 1 ml samples and incubated at 5, 10, 15, 20, 25, 30 or 40°C (3 replicates). The pollen growth showed a broad peak at 15 - 30°C for both percentage of germination and tube growth (Fig. 3.17 & 3.18; See Appendix 1). After 2 hr there was still no germination at 5 and 40°C and a slight germination after 4 and 6 hr incubation at these temperatures.

3.4.6 The effect of pollen density

Pollen from 20 freshly dehisced anthers from 4 flowers of different genotypes was placed in 2 ml SM and adjusted to pH 6.75 with NaOH. From this stock a dilution series was created with 5 pollen densities: approximately 100, 50, 25, 12.5 and 6.25% of the initial densities. Pollen grain density in each sample was then measured on a haemocytometer (3 replicates). The growth was greatest at densities between 6000 - 27000 grains/ml (Fig. 3.19; See

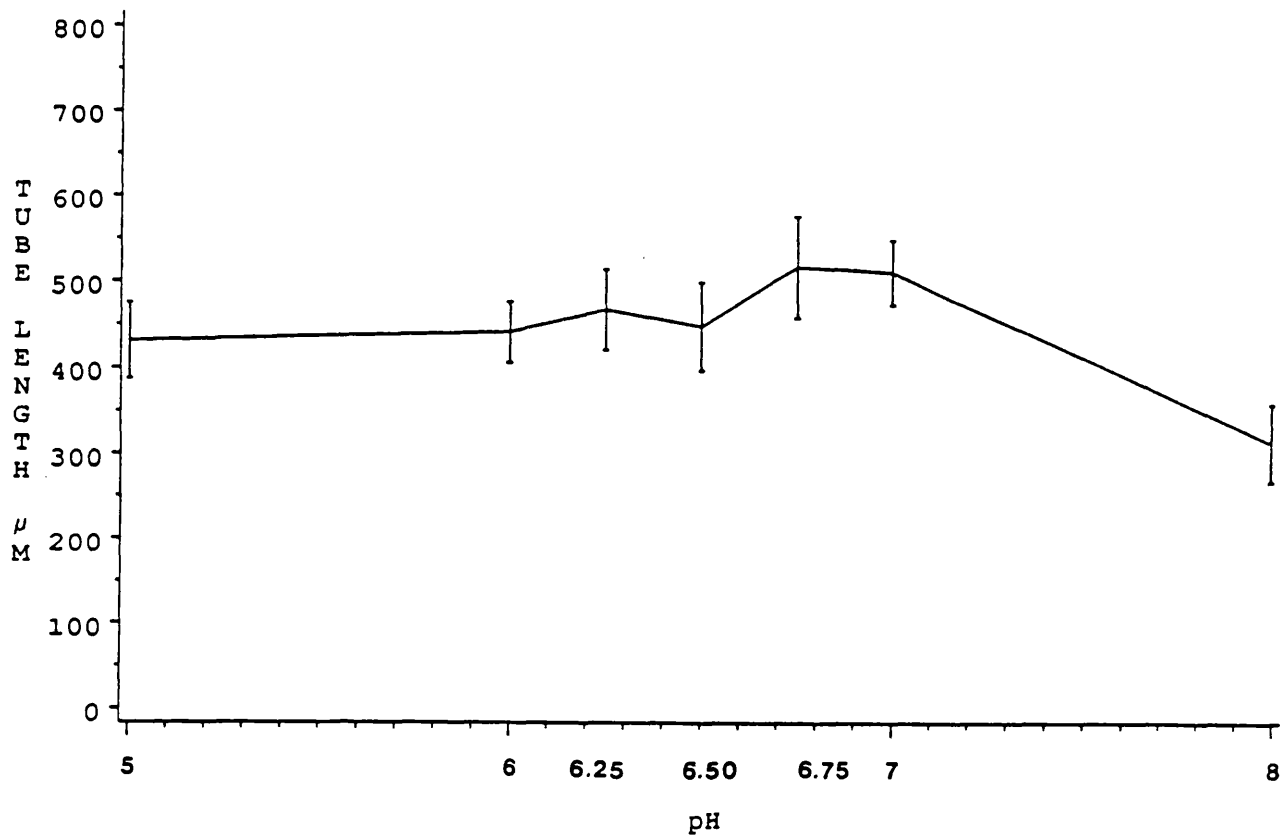


Fig. 3.15 The effect of pH on the tube growth of tobacco pollen after 4 hr (the error bars represent standard deviation).

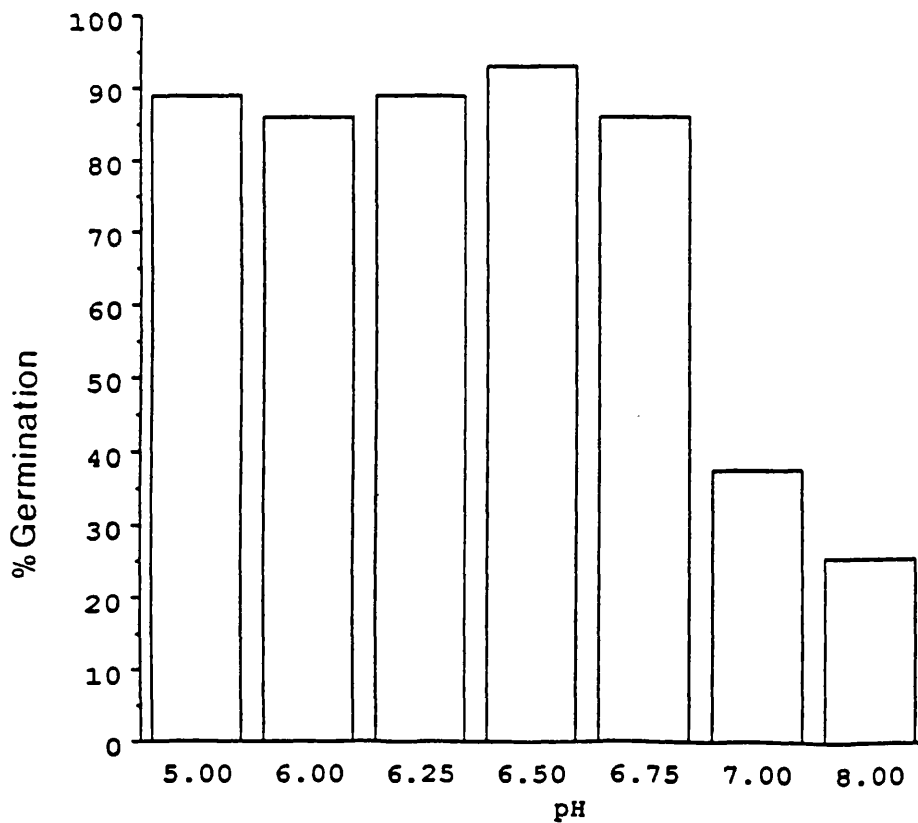


Fig. 3.16 The effect of pH on the % germination of tobacco pollen

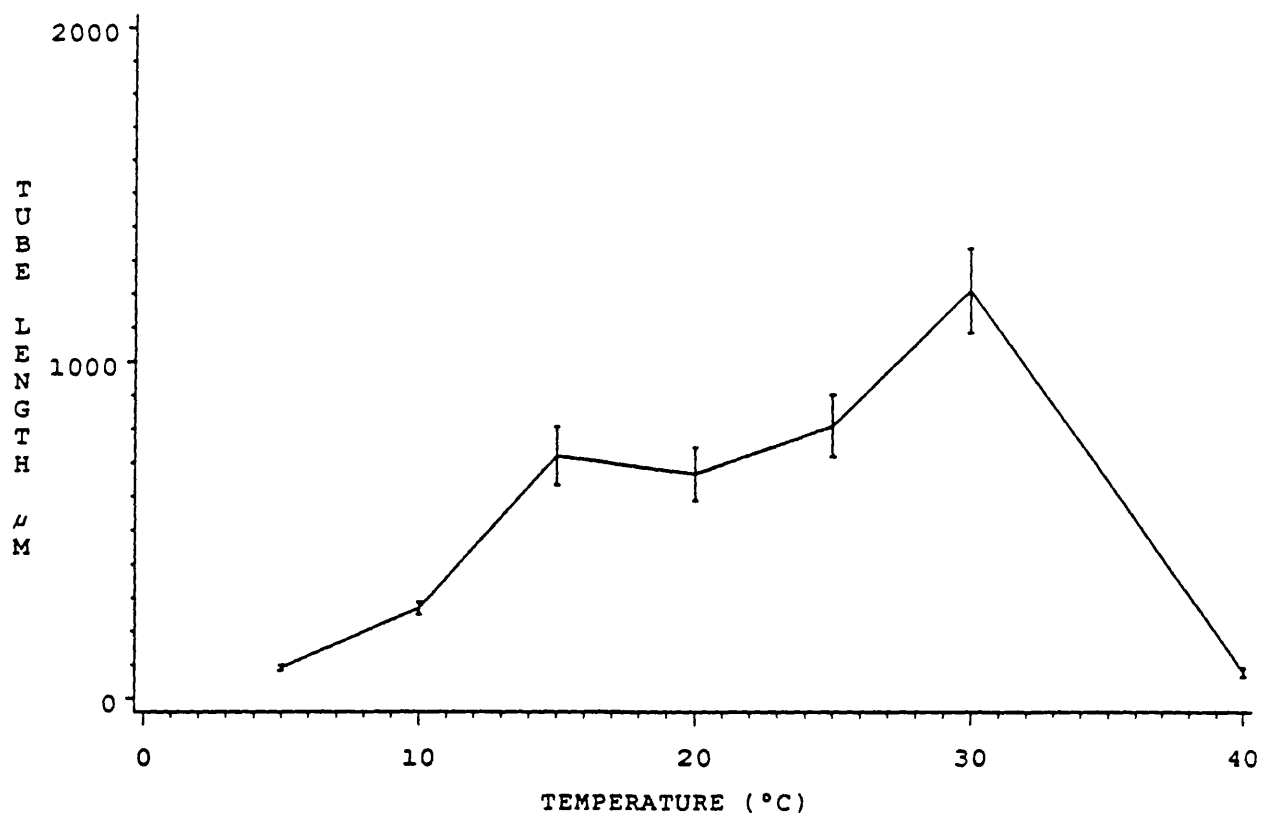


Fig. 3.17 The effect of temperature on the tube growth of tobacco pollen after 4 hr (the error bars represent standard deviation).

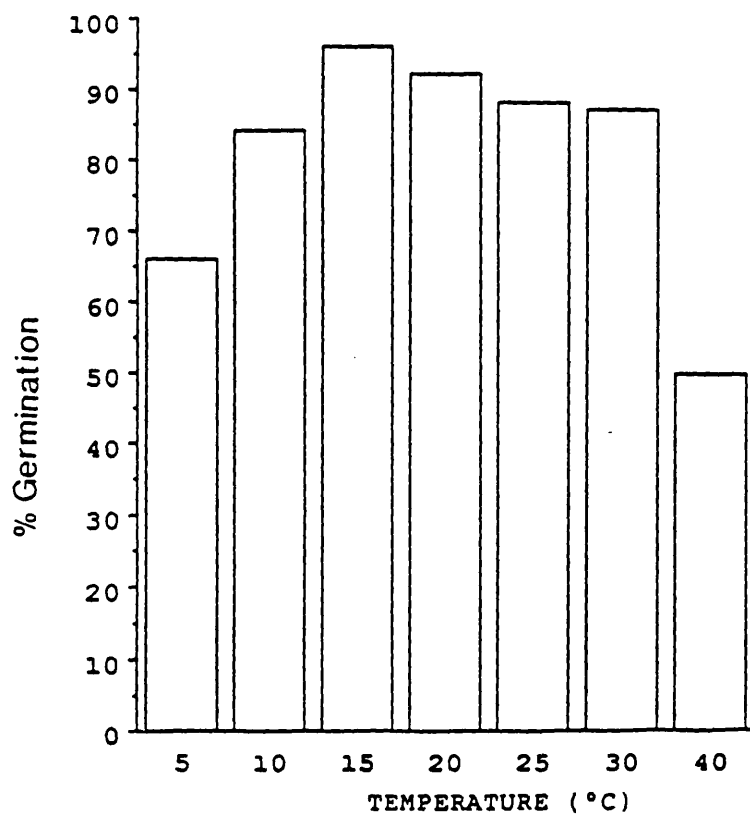


Fig. 3.18 The effect of temperature on the % germination of tobacco pollen

Appendix 1). The percentage germination recorded was equally high at these concentrations (Fig. 3.20). Both tube length and percentage germination were reduced at higher incubation densities.

3.4.7 The effect of shaking

Pollen from 5 freshly dehisced anthers from 2 flowers of one genotype was added to 2 ml of SM then adjusted to pH 6.75 with Tris HCl. After mixing thoroughly, the sample was divided into two halves; one of these was shaken continuously on an orbital shaker whilst the other was placed on a shelf to one side of the machine (3 replicates). There was no significant difference between the shaken and static samples on any treatments (Fig. 3.21; See Appendix 1), and the tubes in both treatments were healthy with no burstage apparent.

3.4.8 Effect of genotypes

Pollen from 5 freshly dehisced anthers from 5 flowers (different genotypes) was added to 5 ml of SM. The pollen was germinated, each anther in 1 ml. There was no significant difference in pollen tubes length (Fig. 3.22; See Appendix 1), and percentage germination between these five genotypes (Fig. 3.23).

3.5 DISCUSSION

SM was the medium of choice for germinating tobacco pollen in spite of the fact that pollen tube growth was slightly better in BM and FM. The advantages of SM is that it lacks mineral salts which may effect some of the

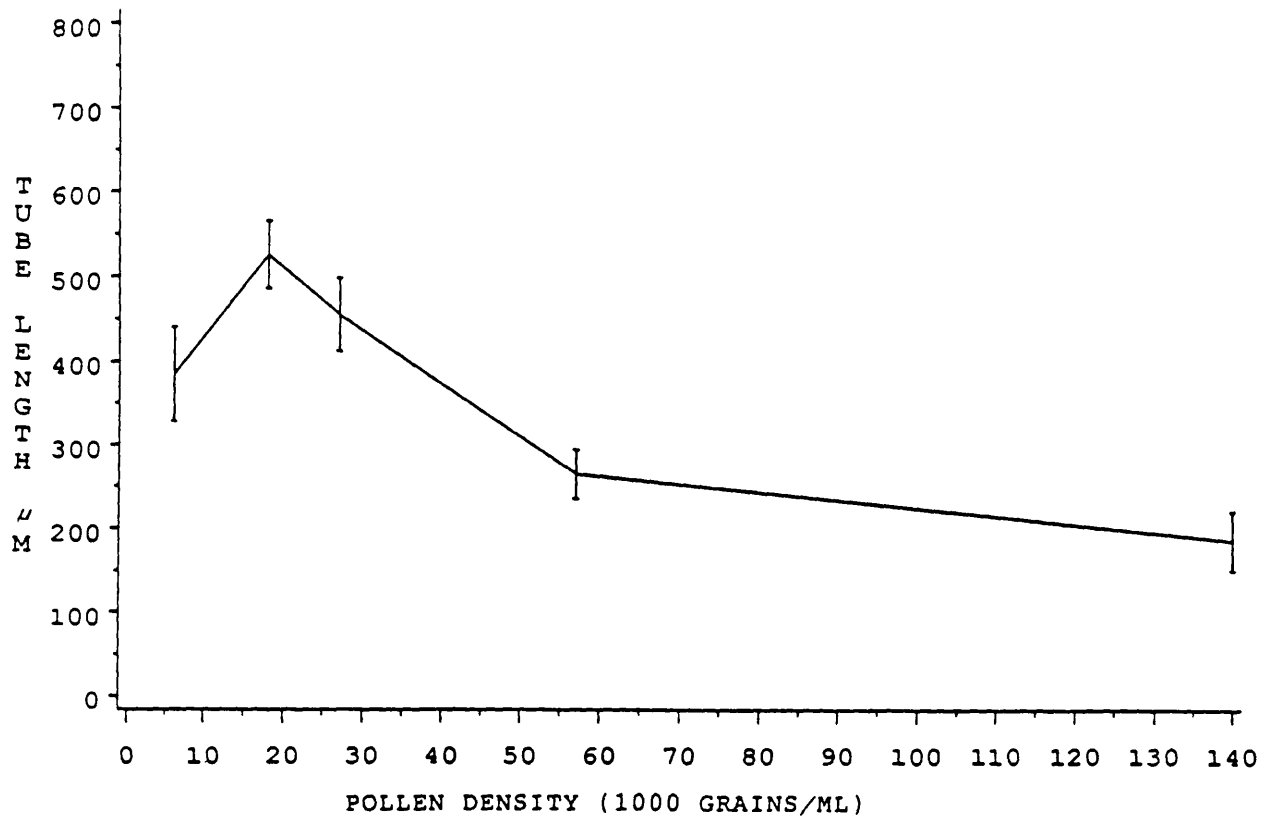


Fig. 3.19 The effect of pollen density on the tube growth of tobacco pollen after 4 hr (the error bars represent standard deviation).

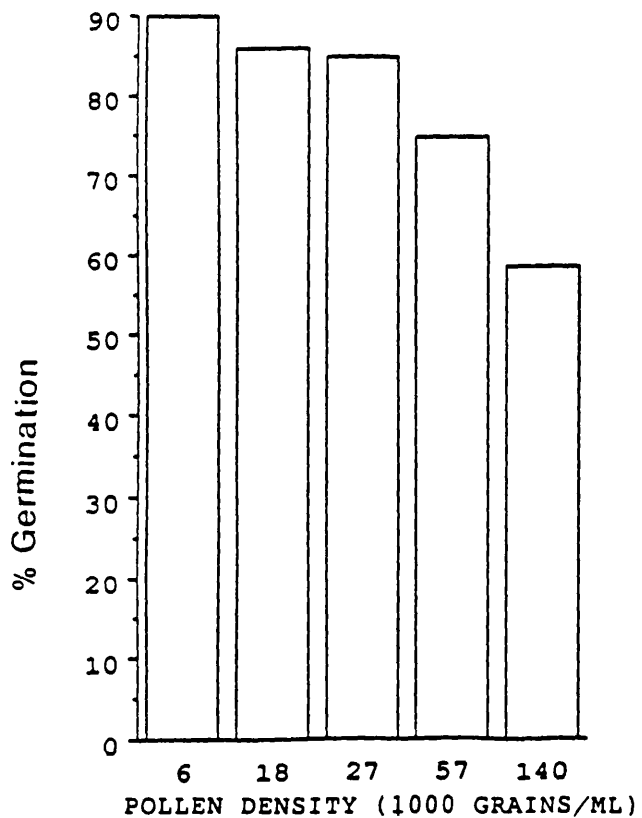


Fig. 3.20 The effect of pollen density on the % germination of tobacco pollen

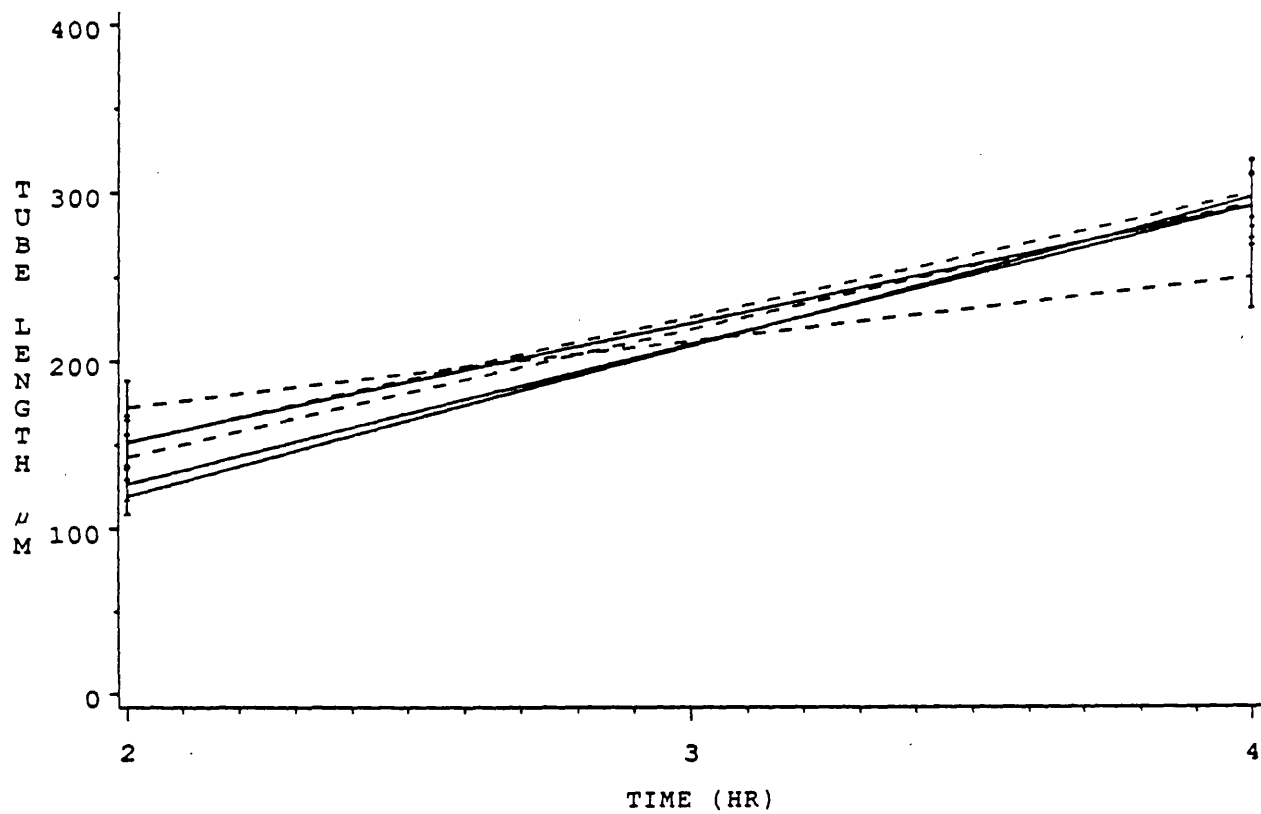


Fig. 3.21 The effect of shaken and static on the tube growth of tobacco pollen after 4 hr (the error bars represent standard deviation).

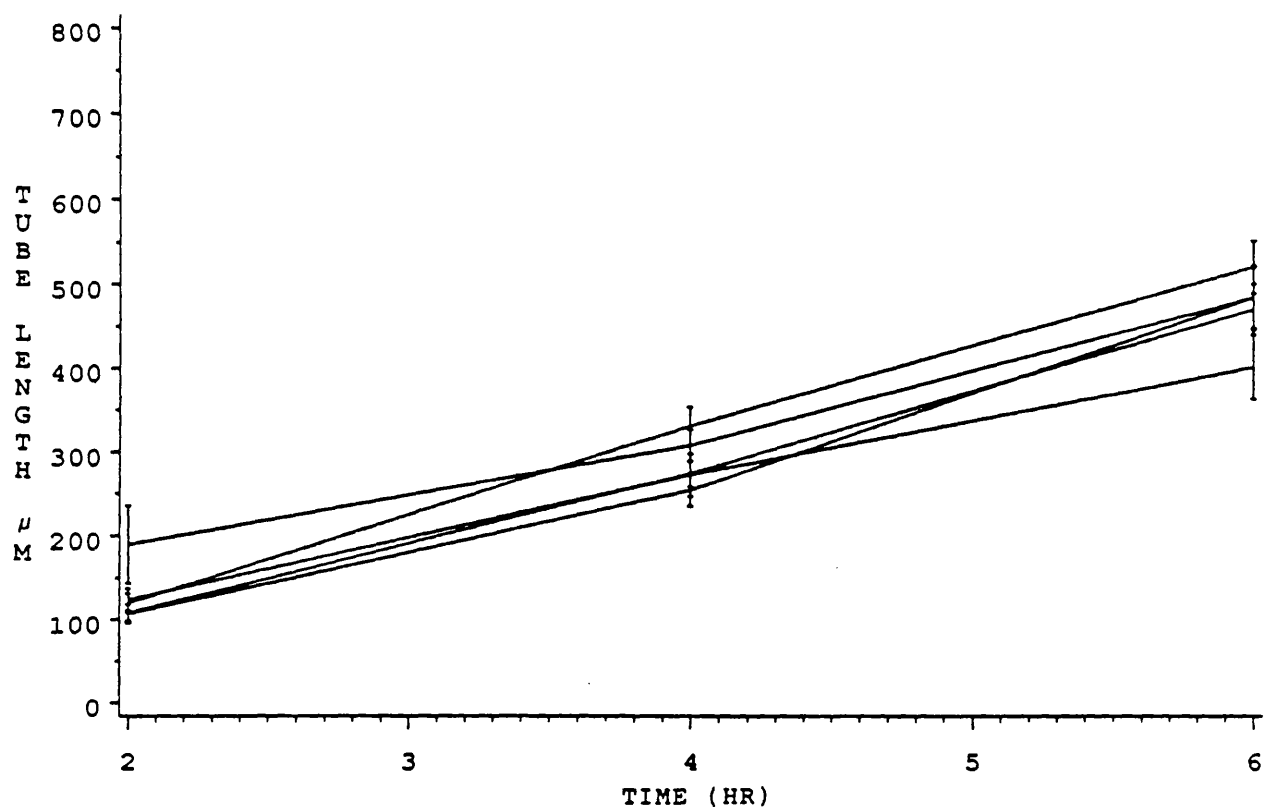


Fig. 3.22 The effect of genotypes on the tube growth of tobacco pollen after 4 hr (the error bars represent standard deviation)

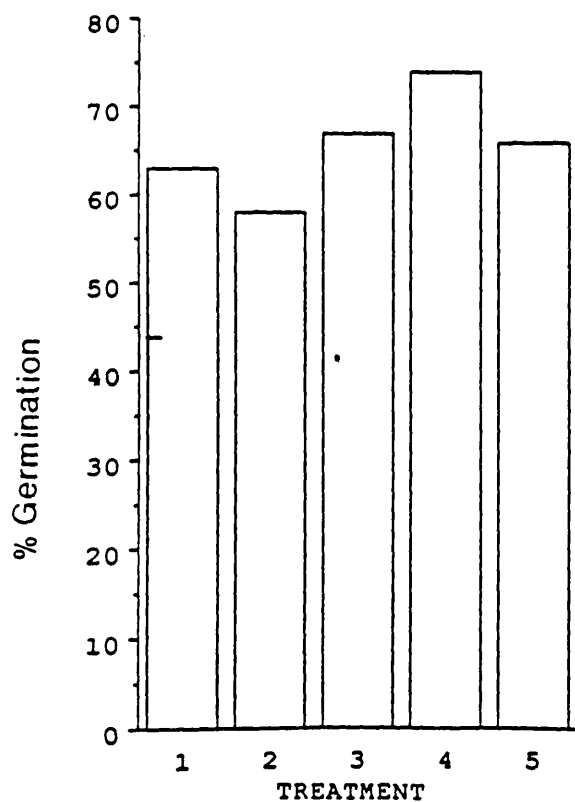


Fig. 3.23 The effect of physiological state on the % germination of tobacco pollen

chemicals which will be used in transformation experiments.

Between 10% and 18% sucrose was observed to generate the best *in vitro* germinated tobacco pollen; the greatest tube length and highest percentage germination both occurred at about 15% sucrose, which differs from many reports, which stated that 10% sucrose is the optimum sucrose concentration (Tupy et al., 1955; O'Kelly, 1955; Heslop-Harrison et al., 1985)

The results with boron concentration agreed with many reports that 100 mg/l was the optimum for pollen germination. The lower concentration of boric acid gave inhibitory results but with high concentrations (200-400 mg/l), there was a similar tube growth to the optimum.

Tobacco pollen was found to be less sensitive to change in temperature than might be expected. Heslop-Harrison germinated tobacco pollen at 24°C and apart from that there is no mention of the optimum temperature in other reports. Thus, 25°C was adopted for all future experiments. There was no difference between pollen germination in shaken or static conditions and so in future all experiments were carried out in static culture.

Incubation at densities 6000 - 27000 grains/ml gave very good percentage germination and excellent tube length. At densities above this both parameters were inhibited. Perhaps competition for nutrients, may have caused this inhibition.

The genotype of an individuals of the plant appeared to play no role at all if the plants were grown in the

same environment. There was no significant difference between the pollen tube length and percentage germination of samples from any particular plant, however pollen germination was poor if the plant was old, infested with aphids or infected with bugs.

3.6 IN VITRO GERMINATION OF PEA POLLEN

The cultivated pea is a commercially important species with bicellular pollen that does not grow very well in culture (Setia & Malik, 1984). Although previous studies on liquid culture systems report a good percentage germination (70 - 100%), pollen tube growth has been observed to cease after 4 - 5 hr, producing maximum lengths of between 200 and 600 μm (Vasil, 1962; Singh, 1972; Setia & Malik, 1984).

There has been some disagreement in the literature over the optimal sucrose concentration required for the germination of pea pollen. Jahr and Gottschalk (1973) and Wolfe (1974) chose 30% sucrose for their agar-based solid media, whereas Bose (1959) selected 1 M (34%) sucrose. Nair and Singh (1972) suggested that the optimal for liquid system was 15% sucrose for germination, but 32% sucrose produced the longest tubes; they used media containing 32% sucrose for subsequent experiments. However, Setia and Malik (1984) stated simply that 'maximum pollen germination and tube growth was noted in media consisting of 15% sucrose and 0.01% boric acid'. Ahokas (1987) chose a liquid medium with 5% sucrose in which to germinate pea pollen in his transfection experiments. Vasil (1962) obtained good germination and

the longest tube lengths of those reported in 25-30% sucrose and 0.01% boric acid. Detailed results were not presented in any of these works.

The stimulatory effect of boron has been known for many years and it is widely used in even the most basic of germination media. The optimal concentration of boron in culture media varies greatly and depends on a range of factors including endogenous borate concentration in the pollen, culture conditions, and on the growth environment of the plant. Usually maximal stimulation of tube growth and germination occurs at concentrations between 10 and 150 mg/l (Visser, 1955; Vasil, 1964). Setia and Malik (1984) stated that 0.01% (100 mg/l) boric acid is optimal for pea pollen germination *in vitro* although evidence was not supplied in support of this assertion.

Setia and Malik (1984) reported that maximum germination and pollen tube growth in the pea occurred between pH 4.5 and 5.5. However, Vasil (1962) had previously obtained equally high germination and greater tube lengths in a medium adjusted to pH 6.6 - 6.8. Setia and Malik (1984) also reported maximal tube growth for pea pollen at 28°C, whereas greatest percentage germination occurred at 25°C. Incubation of pollen above or below these temperatures resulted in both processes being inhibited.

The merits of shaking culture techniques for *in vitro* germination is long established (e.g. Mascarenhas, 1966; Roggen and Stanley, 1969) but to my knowledge has yet to be applied to the culture of pea pollen.

It has been established in many species that pollen

grains cultured in dense and large populations germinate better and form longer tubes than those germinated in sparse populations in the same medium (e.g. Brink, 1924; Brewbaker and Kwack, 1963). This effect has been attributed to the abundance of the calcium cation (Ca^{2+}) in the medium (for review see Vasil, 1987).

Gross changes in environmental variables such as temperature, soil water content, day length, and insect or fungal infection have been implicated in causing pollen sterility in a number of plants. Therefore, more subtle changes in these variables may be expected to affect performance (tube growth and percentage germination) rather than the absolute fertility of the pollen.

3.7 RESULTS

3.7.1 The effect of sucrose

Pollen from freshly dehisced anthers of two cultivars (5 anthers from each flower) was incubated in SM medium containing various concentrations of sucrose and 100 mg/l boric acid. Percentage germination and tube length were recorded after 4 hr incubation (3 replicates). The greatest tube growth in all replicates occurred in media containing 20 or 25% sucrose, although some variation was noted in the size of the peaks (Fig. 3.24). Amalgamation of results from individuals revealed no significant differences in tube length between cultivars, with both showing a broad peak, estimated to perform optimally at 22 - 23% sucrose (Fig. 3.25; See Appendix 1). Similarly, percent germination was highest in media containing between 20 and 25°C sucrose in Orb (Fig. 3.26)

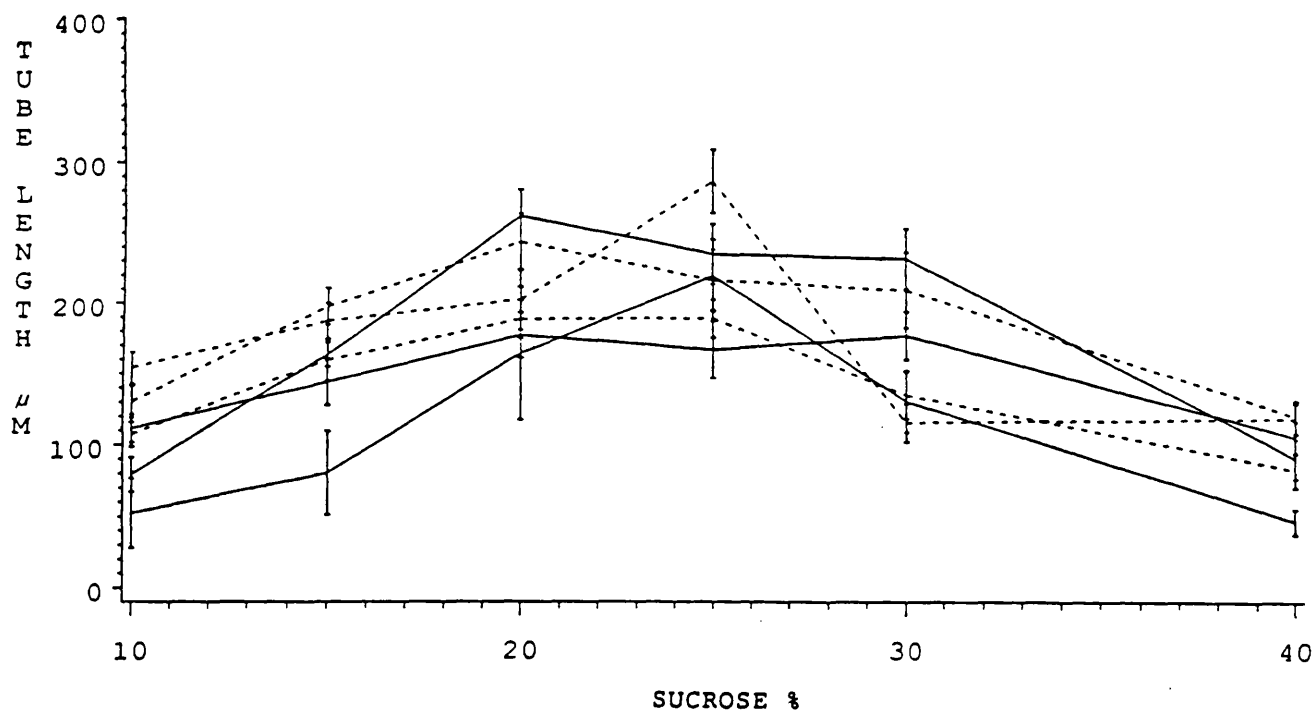


Fig. 3.24 The effect of sucrose on the tube growth of pea pollen after 4 hr. ----- Consort, ——— Orb.

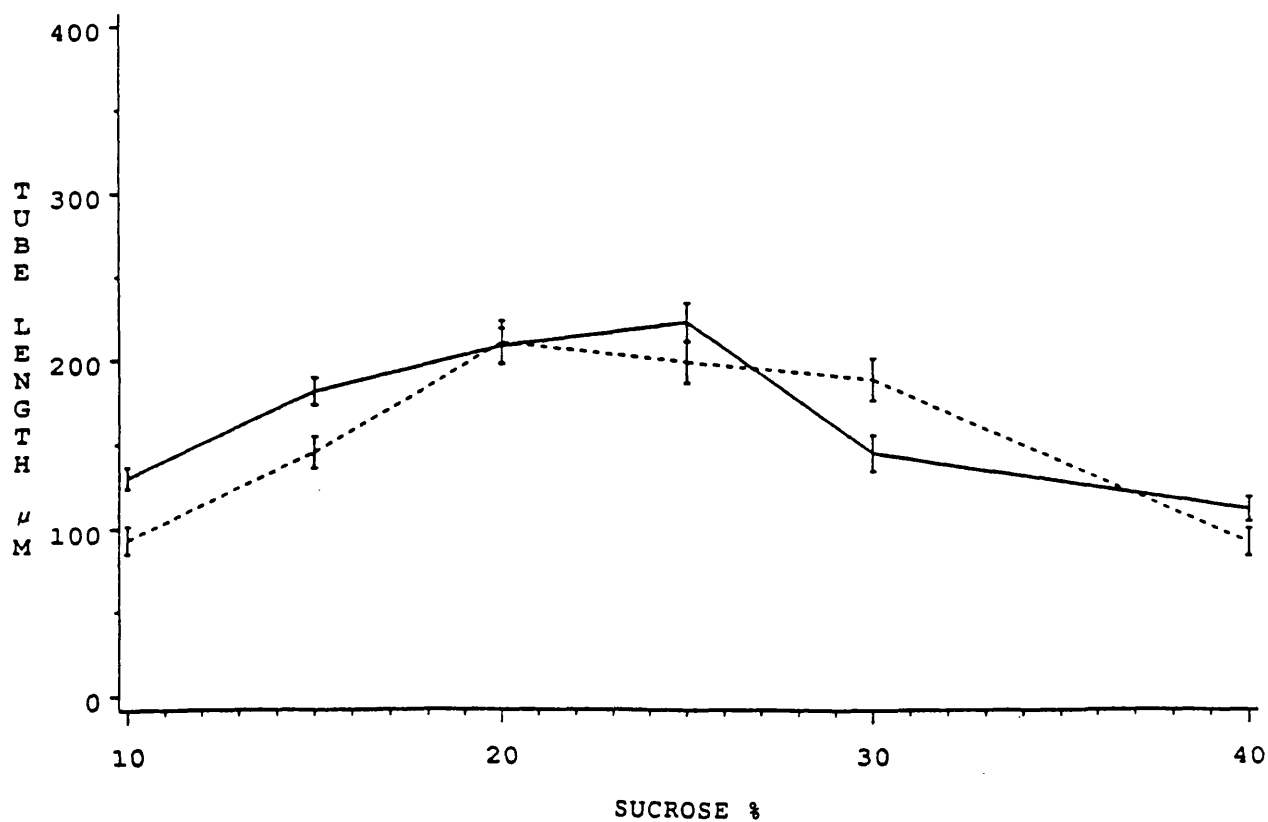


Fig. 3.25 The effect of sucrose on the tube growth of pea pollen (Amalgamation of results of Fig. 3.24)

and Consort (Fig. 3.27). The procedure was then repeated using pollen from cv. Orb in Brewbaker and Kwack medium (BM) and in Franklin-Tong medium (FM). Tube growth was greatly enhanced in both BM and FM in comparison with SM, although the same sucrose concentration (22 - 23%) was optimal. Percentage germination also peaked between 20 and 25% sucrose.

3.7.2 The effect of boron

Pollen from 9 freshly dehisced anthers from 2 flowers of one genotype from cv. Orb was germinated in media containing 23% sucrose and 0, 1, 10, 50, 100, 200, 300, 400, or 500 mg/l boric acid. Pollen tube length and percentage germination (5 replicates) were measured after 4 hr in culture. A slight promotion of tube growth and percentage germination was observed in SM after the addition of 1 - 100 mg/l boric acid over the control which contained no boric acid (Fig. 3.28 & 3.29; See Appendix 1). However, significant differences were detected within this range and above this value boron became inhibitory.

Tube growth and percentage germination were inhibited in BM by concentrations of 1 - 50 mg/l boric acid but was stimulated by 100 mg/l boric acid (Fig. 3.30 & 3.31). The performance of pollen cultured in BM containing 200 - 500 mg/l boric acid was essentially similar to the control (no boric acid). Accordingly, a concentration of 100 mg/l boric acid was selected for all subsequent experiments.

Thus, the effect of boron on pollen germination in BM appears to be different from its effect in SM. In the

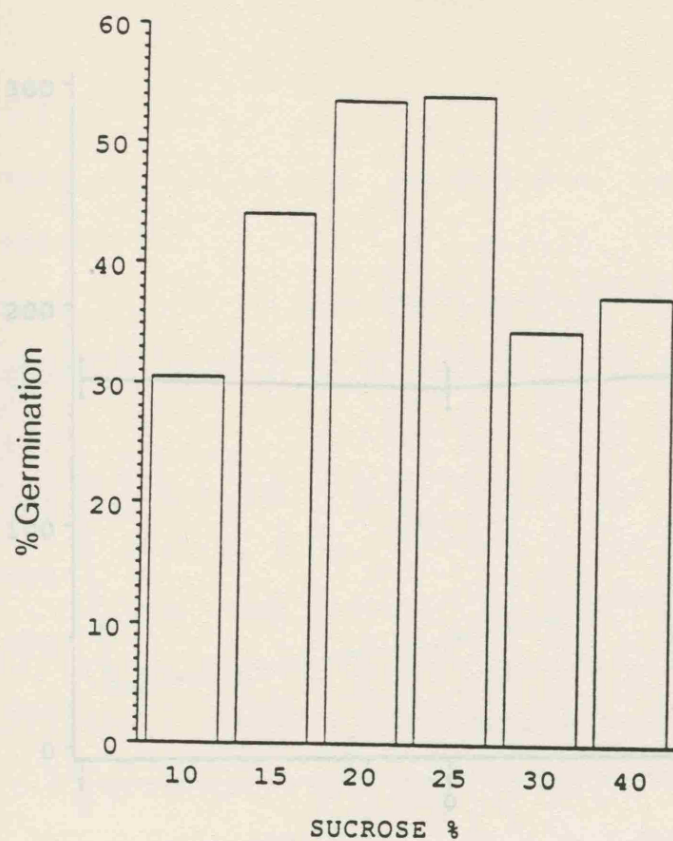


Fig. 3.26 The effect of sucrose on the % germination of pea pollen (cv. Orb)

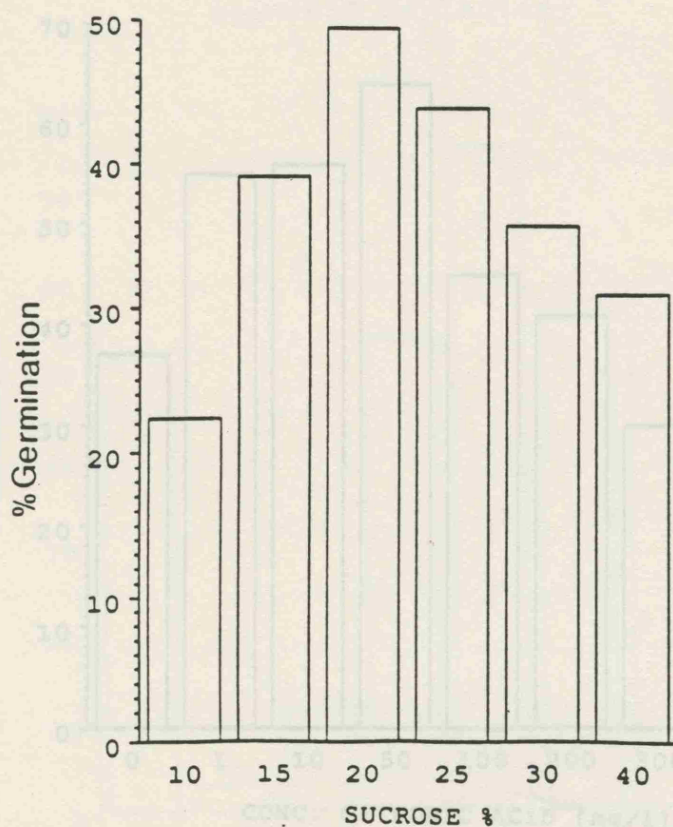


Fig. 3.27 The effect of sucrose on the % germination of pea pollen (cv. Consort)

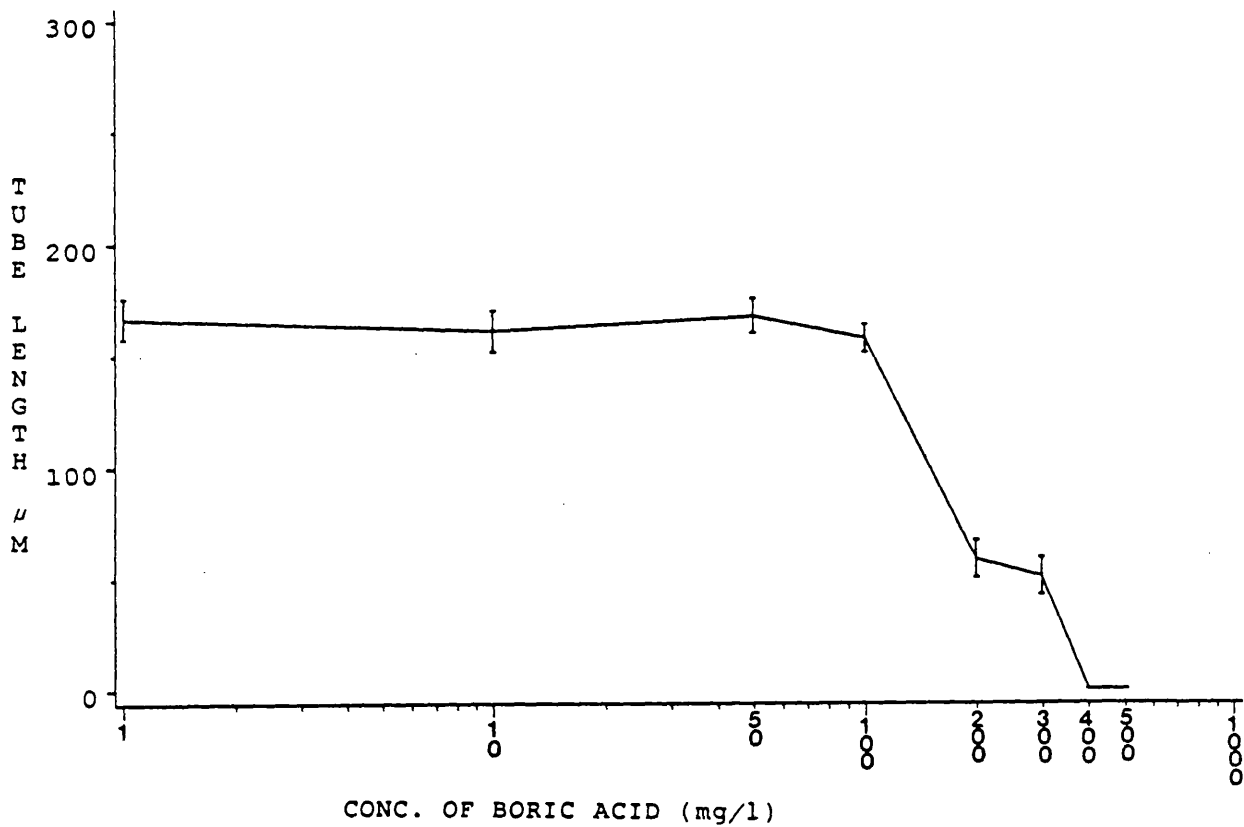


Fig. 3.28 The effect of boric acid on the tube growth of pea pollen (SM) after 4 hr (the error bars represent standard deviation)

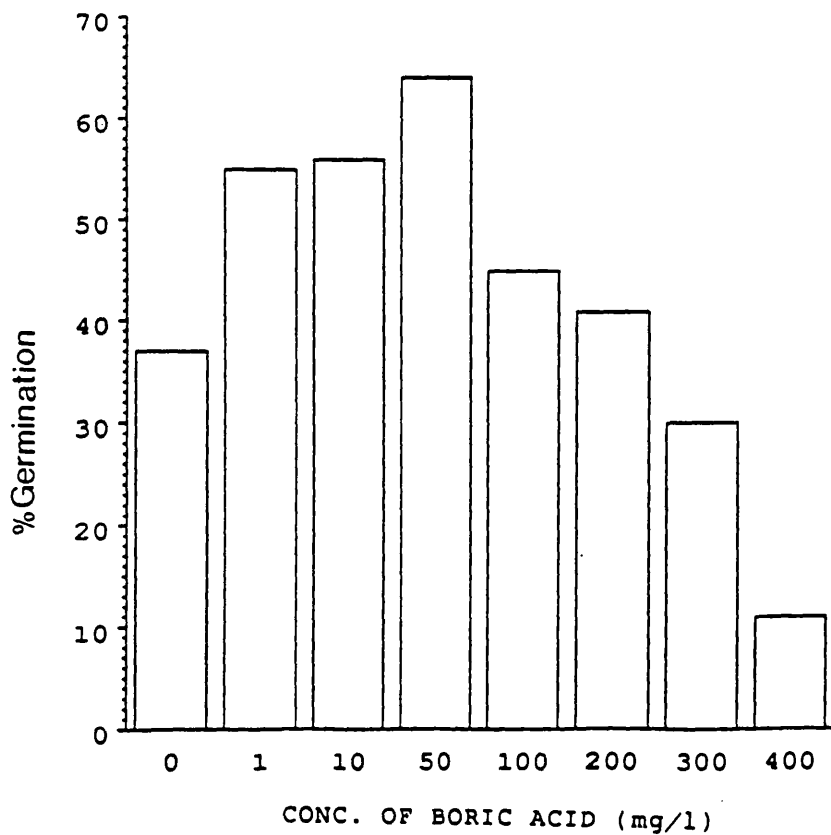


Fig. 3.29 The effect of boric acid on the % germination of pea pollen (SM)

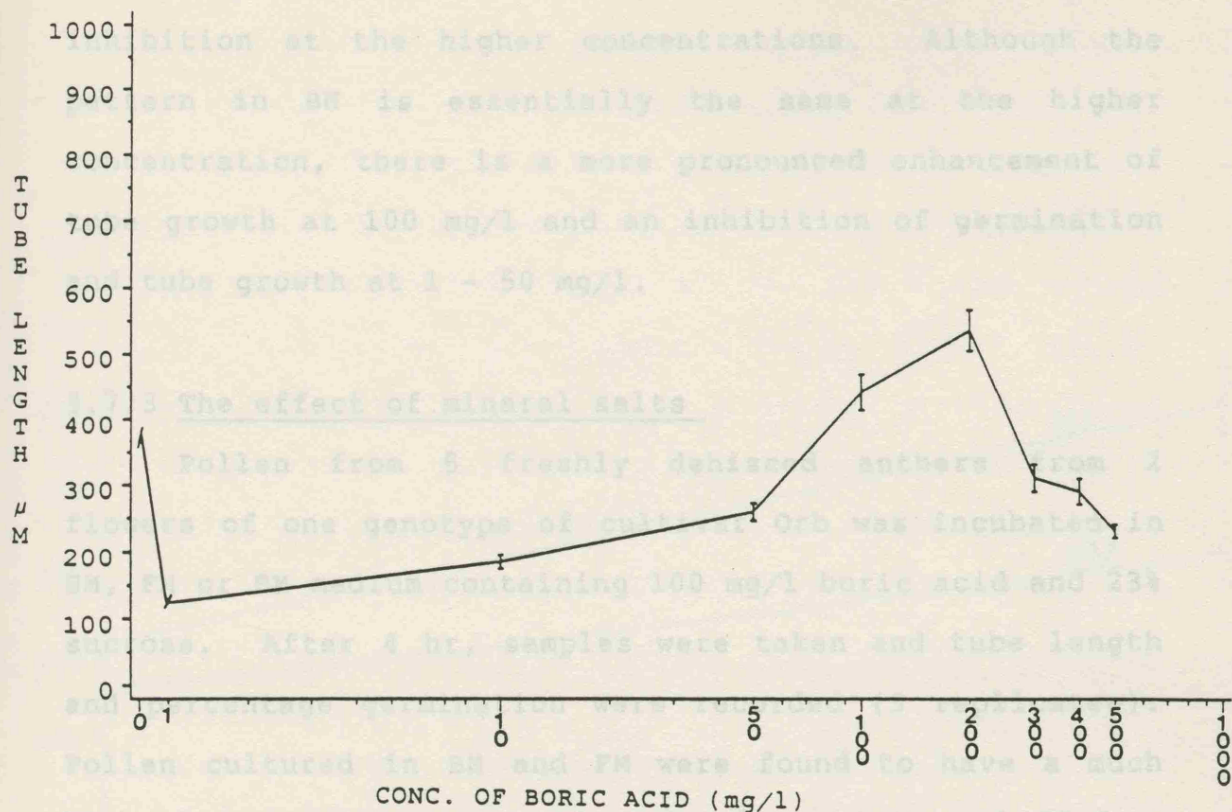


Fig. 3.30 The effect of boric acid on the tube growth of pea pollen (BM) after 4 hr (the error bars represent standard deviation)

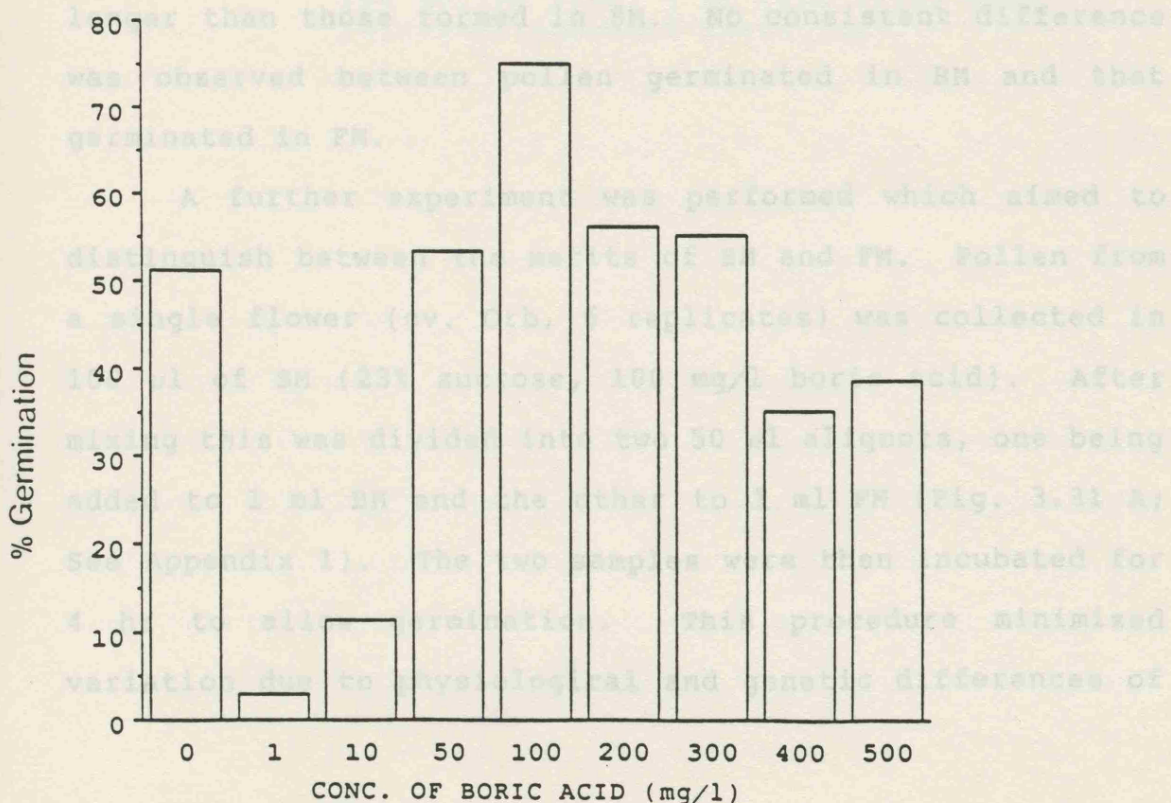


Fig. 3.31 The effect of boric acid on the % germination of pea pollen (BM)

latter there is a slight enhancement of tube growth at relatively low concentrations (1 - 100 mg/l) but an inhibition at the higher concentrations. Although the pattern in BM is essentially the same at the higher concentration, there is a more pronounced enhancement of tube growth at 100 mg/l and an inhibition of germination and tube growth at 1 - 50 mg/l.

3.7.3 The effect of mineral salts

Pollen from 5 freshly dehisced anthers from 2 flowers of one genotype of cultivar Orb was incubated in SM, FM or BM medium containing 100 mg/l boric acid and 23% sucrose. After 4 hr, samples were taken and tube length and percentage germination were recorded (3 replicates). Pollen cultured in BM and FM were found to have a much higher percentage germination (62.5 - 92.4% and 75.2 - 86.2% respectively) than that cultured in SM (32 - 49%). Similarly tubes formed in BM and FM were significantly longer than those formed in SM. No consistent difference was observed between pollen germinated in BM and that germinated in FM.

A further experiment was performed which aimed to distinguish between the merits of BM and FM. Pollen from a single flower (cv. Orb, 6 replicates) was collected in 100 μ l of SM (23% sucrose, 100 mg/l boric acid). After mixing this was divided into two 50 μ l aliquots, one being added to 1 ml BM and the other to 1 ml FM (Fig. 3.31 A; See Appendix 1). The two samples were then incubated for 4 hr to allow germination. This procedure minimized variation due to physiological and genetic differences of

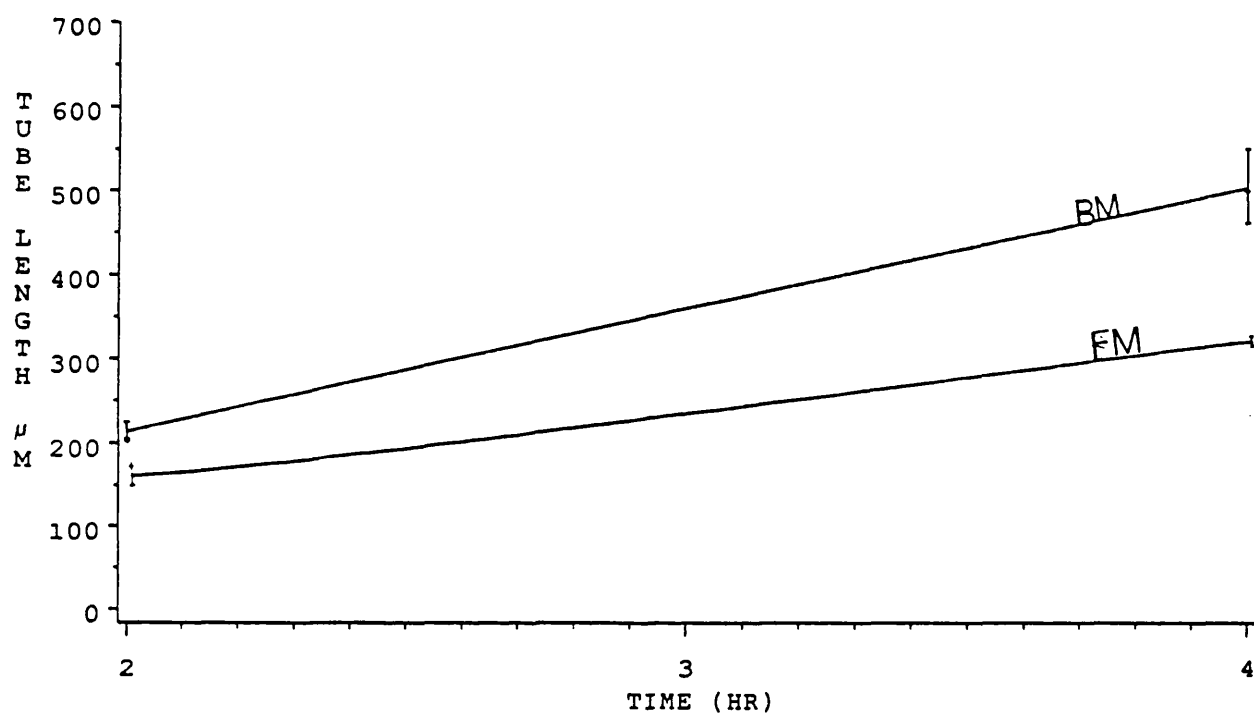


Fig. 3.31 (A) The effect of BM and FM on the tube growth of pea pollen after 4 hr (the error bars represent standard deviation).

different pollen samples and so allowed a more direct comparison of FM and BM to be made. No consistent differences were observed between samples, either in mean pollen tube length or in the percentage germination.

The role of calcium was investigated in a further experiment. Pollen from freshly dehisced anthers of 2 flowers were collected in 100 μ l SM, mixed and divided equally into 1 ml BM without calcium and 1 ml BM supplemented with $\text{Ca}(\text{NO}_3)_2$. No significant differences were observed in the tube lengths of any of the 4 replicates performed and no consistent differences were observed in their percentage germination.

3.7.4 The effect of pH

Pollen from 7 freshly dehisced anthers from 2 flowers (cv. Orb) was placed into 400 μ l of BM adjusted to pH 6.5 with 0.1 M NaOH/HCl. The solution was mixed and 50 μ l aliquots (3 replicates) added to 1 ml samples of BM set to pH 5, 6, 6.25, 6.5, 6.75, 7 or 8 with NaOH/HCl. Where necessary, the pH was readjusted to the original setting after adding the aliquots of pollen. Pollen tube length and percentage germination was recorded from 30 ml samples taken after 4 hr in culture. Tube growth was greatest in media adjusted to pH 6.5 - 6.75 in all replicates (Fig. 3.32; See Appendix 1). Mean tube lengths observed in media adjusted to pH 5 were 32 - 55% of those obtained at pH 6.75. Little difference was observed in the percentage germination of pollen in media set to pH 5 - 6.5, although some inhibition of germination was observed in media of higher pH values (Fig. 3.33). A series of direct

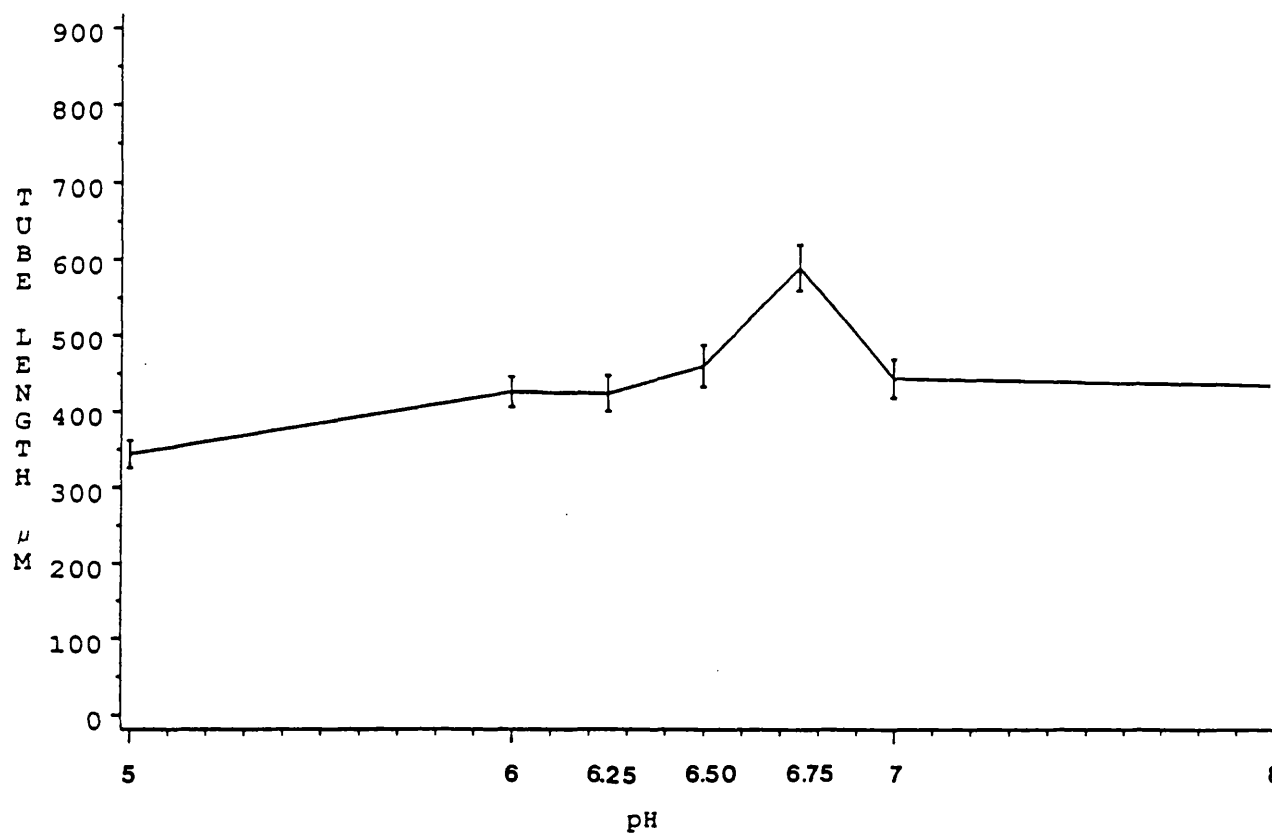


Fig. 3.32 The effect of pH on the tube growth of pea pollen. after 4 hr (the error bars represent standard deviation)

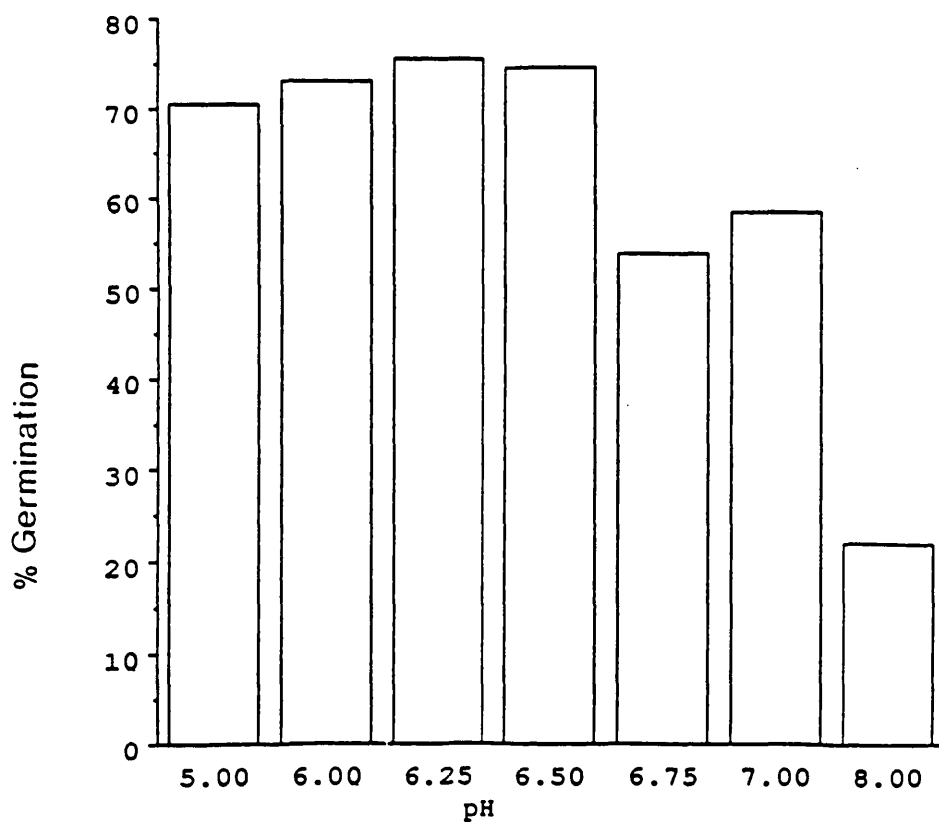


Fig. 3.33 The effect of pH on the % germination of pea pollen

comparisons between the performance of pollen in BM set to pH 6.5 with Tris HCl and that obtained in BM set with NaOH/HCl, revealed no significant differences in tube length (Fig 3.33 A; See Appendix 1) or percentage germination. Tris HCl was used to set the pH of media to pH 6.5 in all subsequent experiments.

3.7.5 The effect of temperature

Pollen from 6 freshly dehisced anthers from 3 flowers of one genotype was placed in 6 ml BM. The solution was mixed, divided into 1 ml samples and incubated at 5, 10, 15, 20, 25, 30 or 40°C (3 replicates). Percentage germination and tube length were recorded after 2 hr and 24 hr. All replicates showed a broad optimal peak of 15 - 25°C for both pollen tube length and percentage germination after 2 hr and 24 hr (Fig. 3.34 & 3.35; See Appendix 1). These temperatures agree well with the day time field temperatures ordinarily encountered by *Pisum* in the UK during the flowering season.

3.7.6 The effect of shaking

Pollen from 5 freshly dehisced anthers from 2 flowers of one genotype (cv. Orb) was added to 2 ml BM. After mixing thoroughly, the sample was divided into two equal halves. One of these was shaken continuously on an orbital shaker whilst the other was placed to one side of the machine. Tube length, percent germination and tube appearance was recorded from 20 μ l aliquots taken after 2 hr and 24 hr incubation (3 replicates). Clear differences were observed between the two treatments in each of the

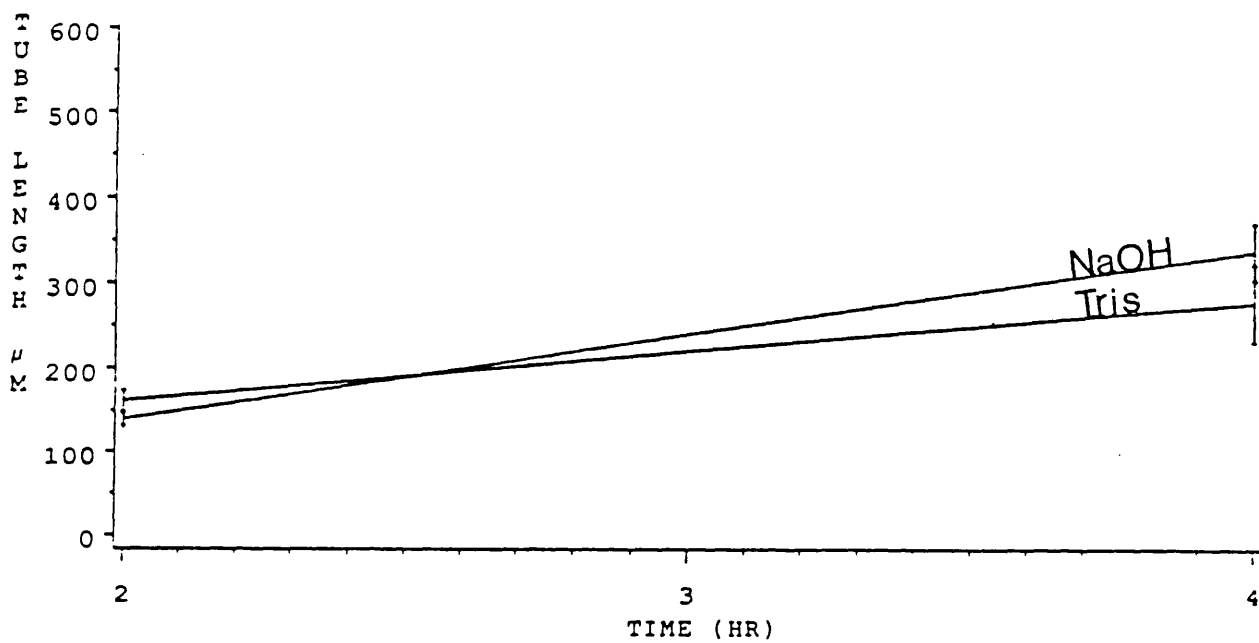


Fig. 3.33 (A) The effect of NaOH/HCl or Tris HCl on the tube growth of pea pollen after 4 hr (the error bars represent standard deviation).

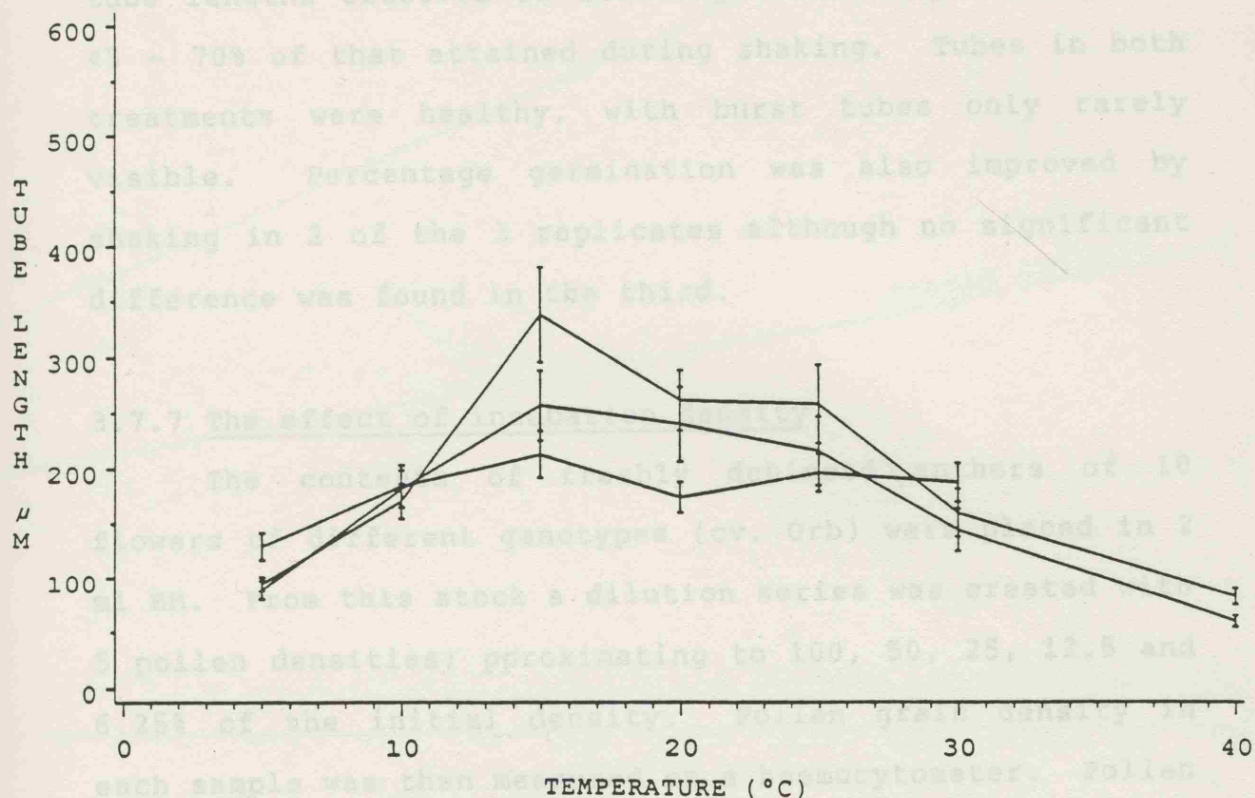


Fig. 3.34 The effect of temperature on the tube growth of pea pollen (SM) after 2 hr (the error bars represent standard deviation)

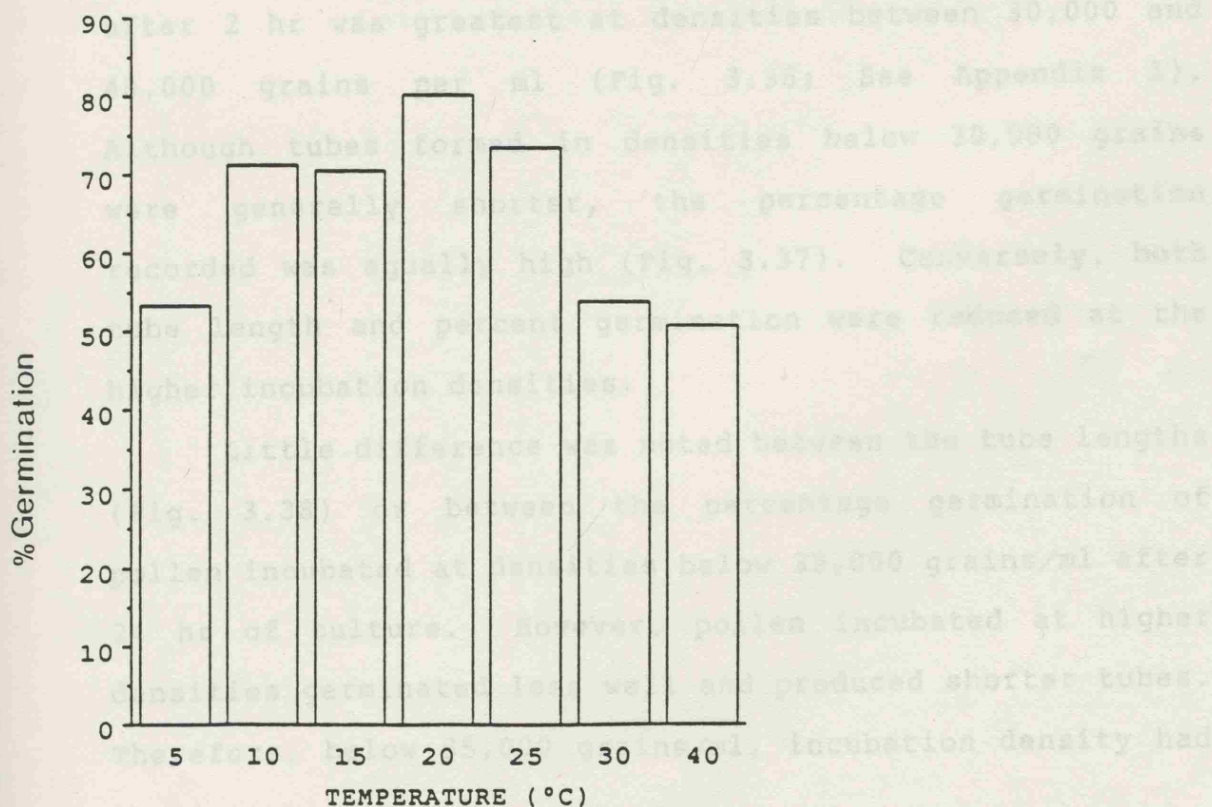


Fig. 3.35 The effect of temperature on the % germination of pea pollen

replicates (Fig. 3.35 A; See Appendix 1). Indeed, mean tube lengths observed in standing media only amounted to 45 - 70% of that attained during shaking. Tubes in both treatments were healthy, with burst tubes only rarely visible. Percentage germination was also improved by shaking in 2 of the 3 replicates although no significant difference was found in the third.

3.7.7 The effect of incubation density

The contents of freshly dehiscent anthers of 10 flowers of different genotypes (cv. Orb) were placed in 2 ml BM. From this stock a dilution series was created with 5 pollen densities; approximating to 100, 50, 25, 12.5 and 6.25% of the initial density. Pollen grain density in each sample was then measured on a haemocytometer. Pollen tube length and percentage germination was recorded after 2 hr and 24 hr in culture (3 replicates). Tube growth after 2 hr was greatest at densities between 30,000 and 40,000 grains per ml (Fig. 3.36; See Appendix 1). Although tubes formed in densities below 30,000 grains were generally shorter, the percentage germination recorded was equally high (Fig. 3.37). Conversely, both tube length and percent germination were reduced at the higher incubation densities.

Little difference was noted between the tube lengths (Fig. 3.38) or between the percentage germination of pollen incubated at densities below 30,000 grains/ml after 24 hr of culture. However, pollen incubated at higher densities germinated less well and produced shorter tubes. Therefore, below 35,000 grains/ml, incubation density had

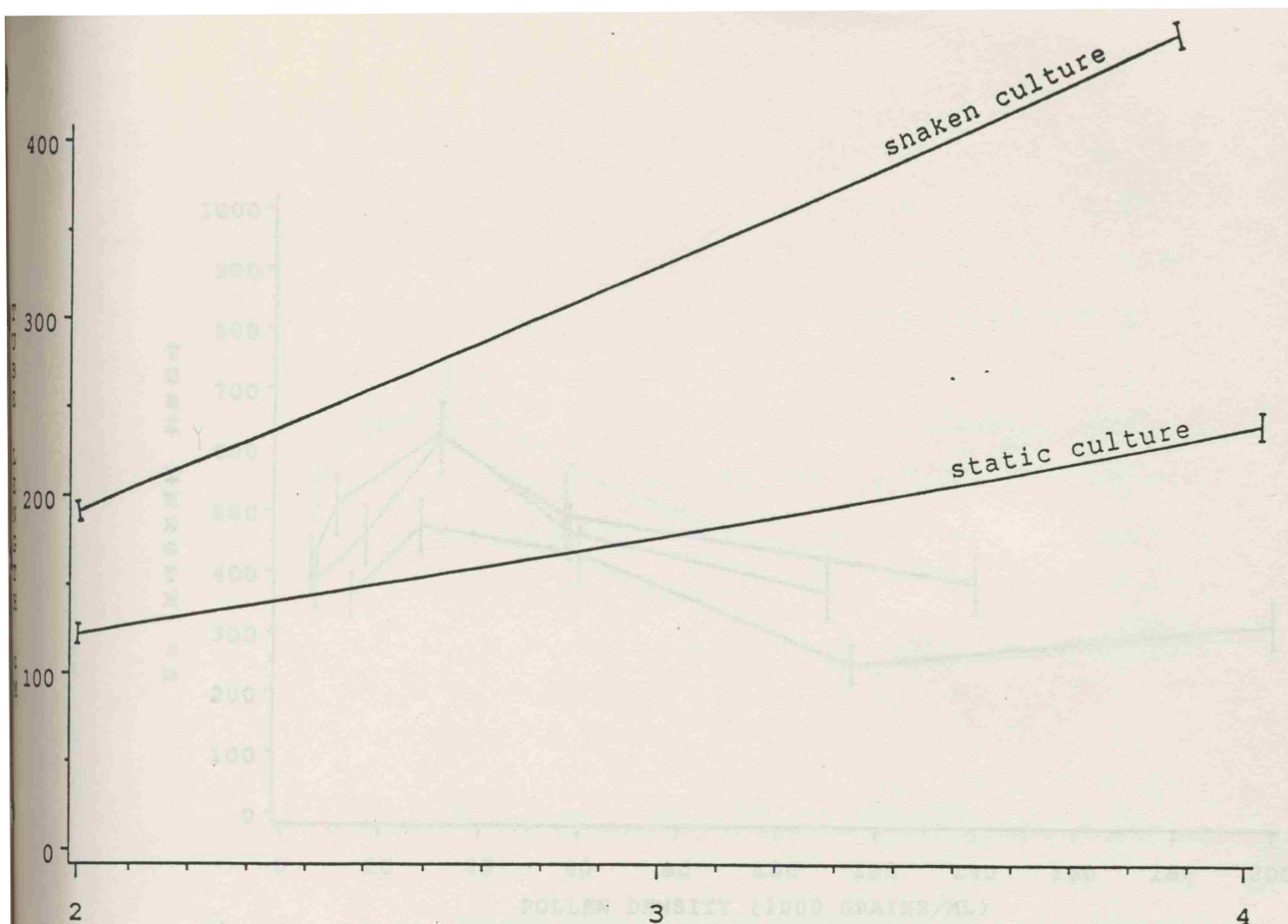


Fig. 3.35 The effect of pollen density on the tube growth of pea pollen after 4 hr (the error bars represent standard deviation).

Fig. 3.35 (A) The effect of shaken and static culture on the tube growth of pea pollen after 4 hr (the error bars represent standard deviation).

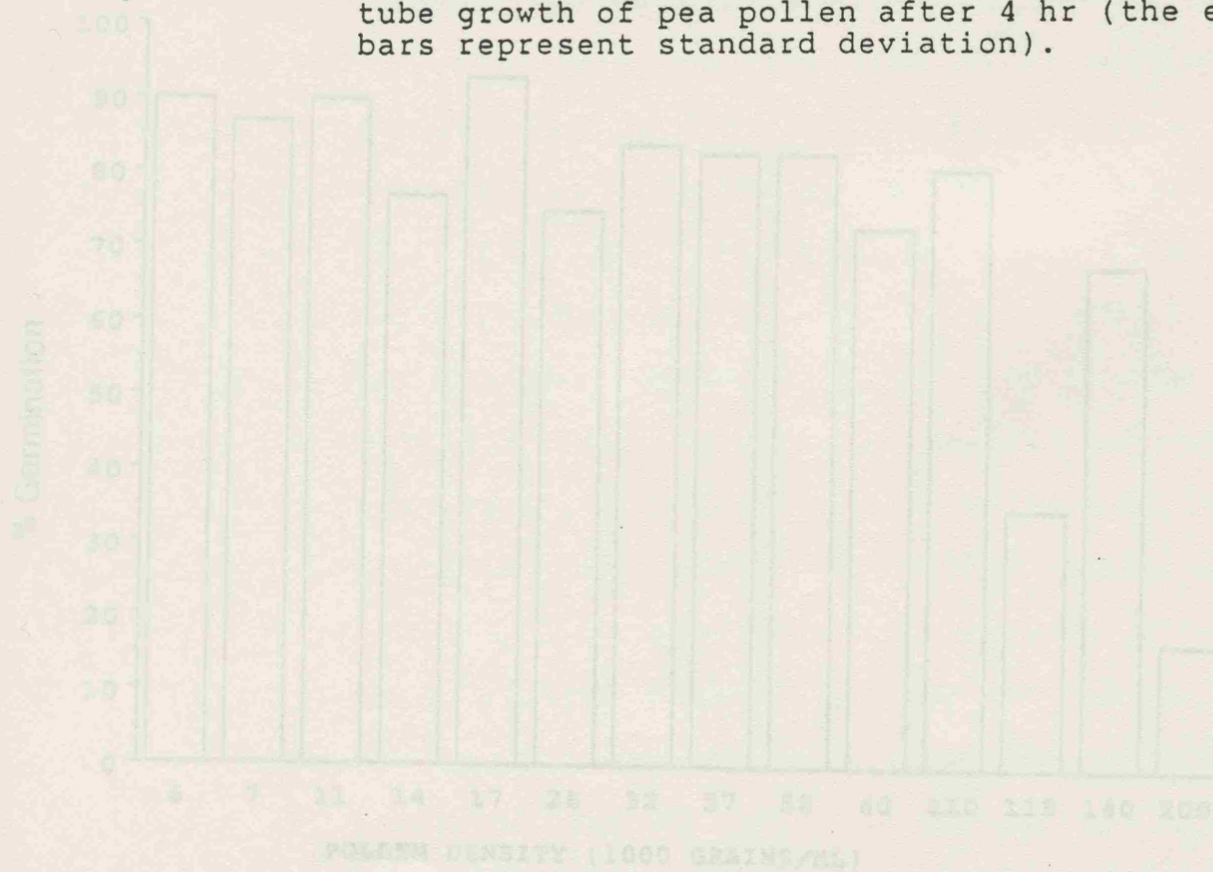


Fig. 3.37 The effect of pollen density on the percentage of germination.

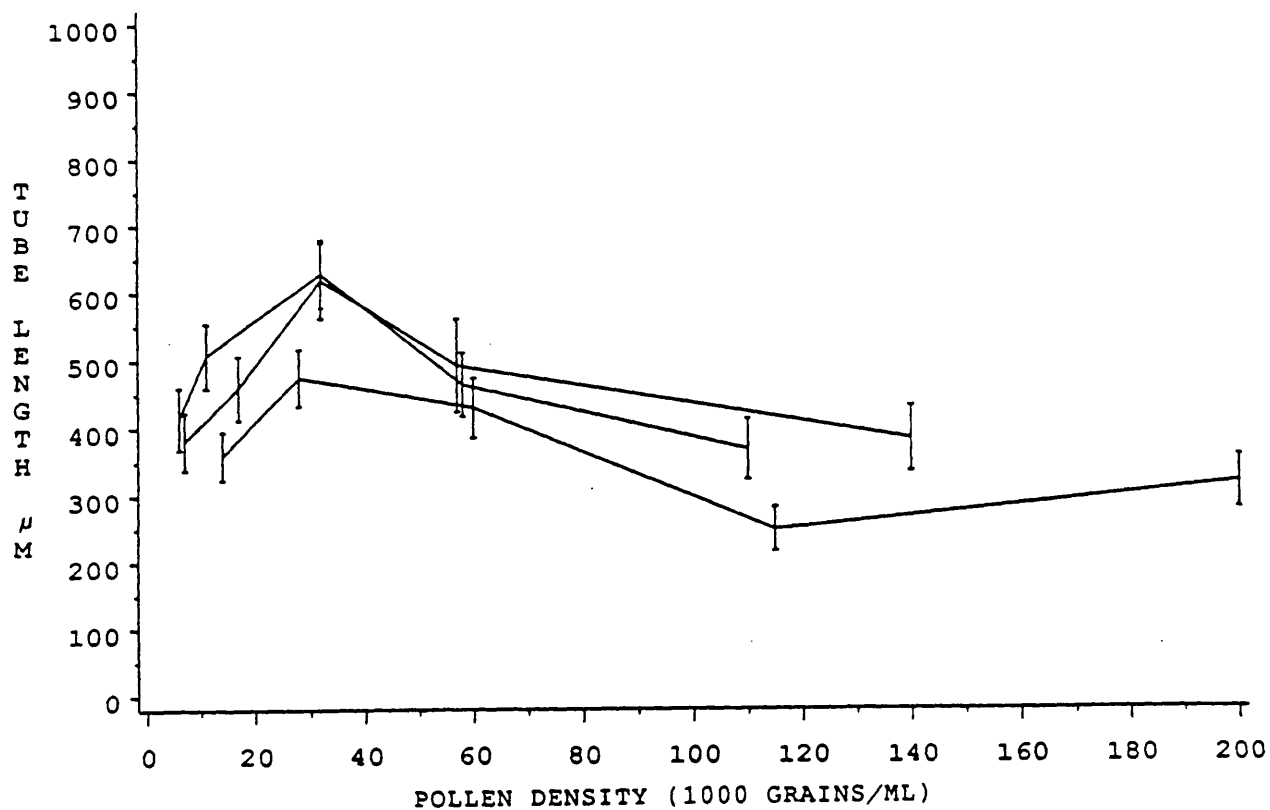


Fig. 3.36 The effect of pollen density on the tube growth of pea pollen after 4 hr (the error bars represent standard deviation).

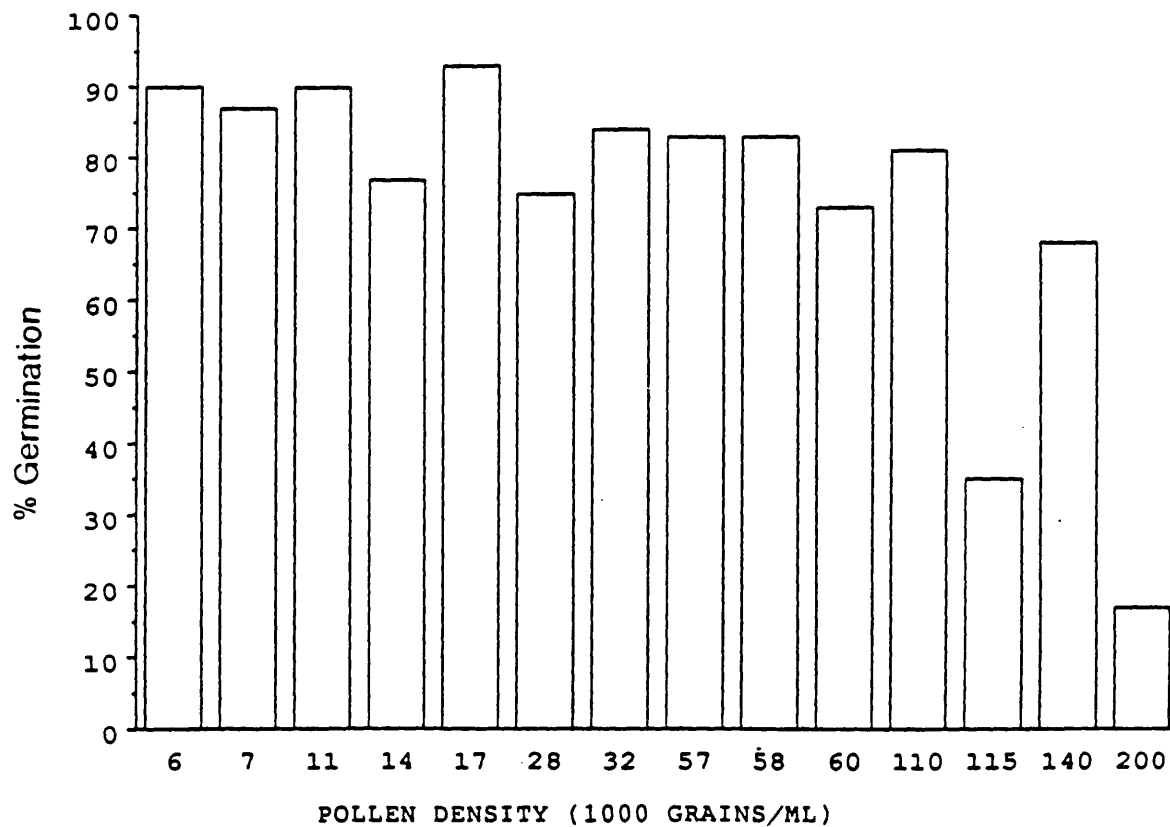


Fig. 3.37 The effect of pollen density on the % germination of pea pollen (After 2 hr)

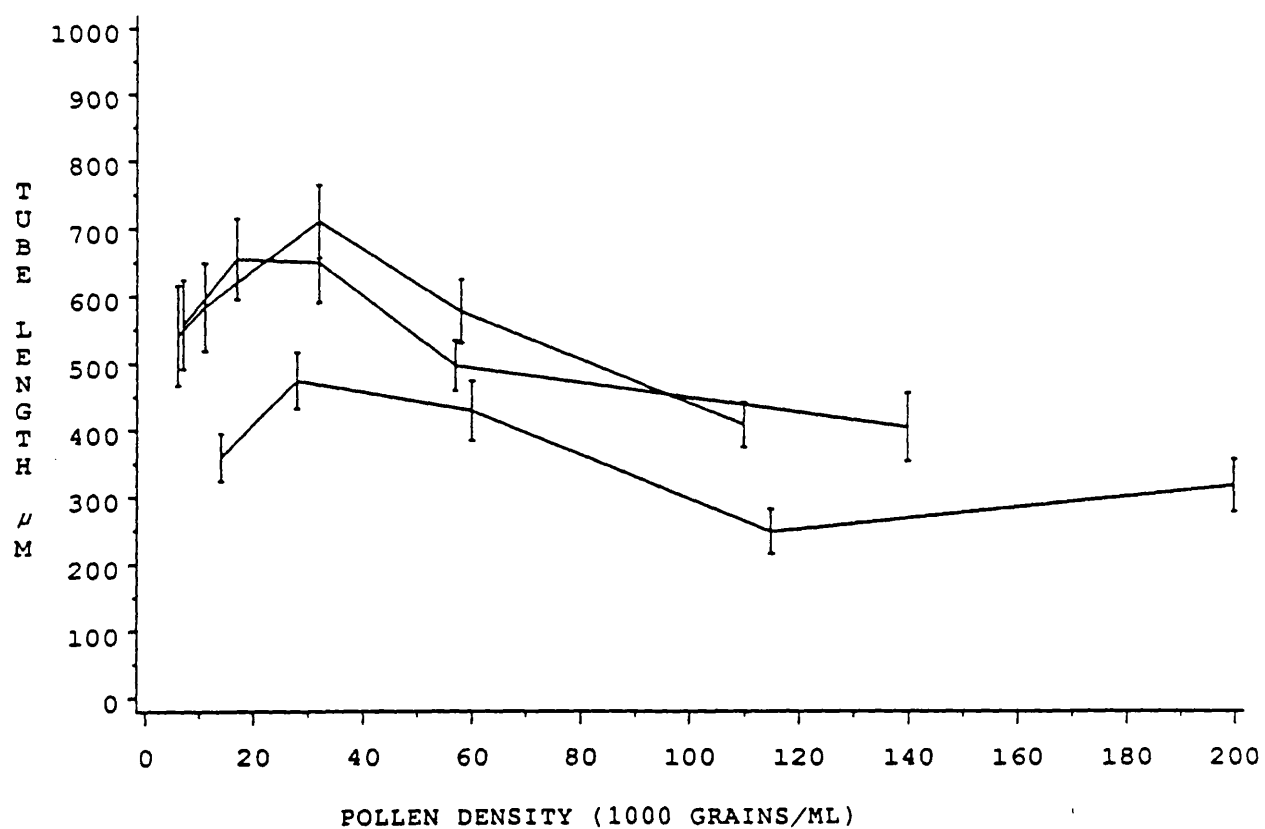


Fig. 3.38 The effect of pollen density on the tube growth of pea pollen after 24 hr (the error bars represent standard deviation).

little effect on the performance of pea pollen in BM other than on initial tube growth rate; densities of 25,000 to 35,000 grains/ml seemingly producing more rapid growth whilst germination and tube growth were inhibited at the higher densities.

Given that rapid growth is desirable for the fertilisation of pea from culture pollen (See section; 3.9.2) and is also likely to be preferred for work on gametophyte selection, it was decided that an incubation density of 25 - 35,000 grains/ml (approx. 1 flower/ml) would be used in subsequent experiments.

3.7.8 Germination under 'optimal' conditions

Pollen from 20 freshly dehiscent anthers from 5 flower of different genotypes (cv. Orb) or (cv. Consort) was placed in 1 ml BM. The sample was incubated at 25°C on an orbital shaker for 24 hr. Pollen tube length and percentage germination were measured from 30 ml aliquots (6 replicates) taken after 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6 and 24 hr in culture. No lag period was detected in either Orb (Fig. 3.39 & 3.40; See Appendix 1) or Consort (Fig. 3.41 & 3.42; See Appendix 1), with significant tube growth being found in all replicates after only 30 min in culture. Tube growth rate was rapid and nearly constant in all replicates during the initial 4 hr of culture but approached zero by the sixth hour. No further growth was observed after this time. Final mean tube lengths of 500 - 1300 μm and 500 - 1500 μm were obtained for cultivars Orb and Consort respectively. Some germination occurred in most replicates within 30 min of the pollen being

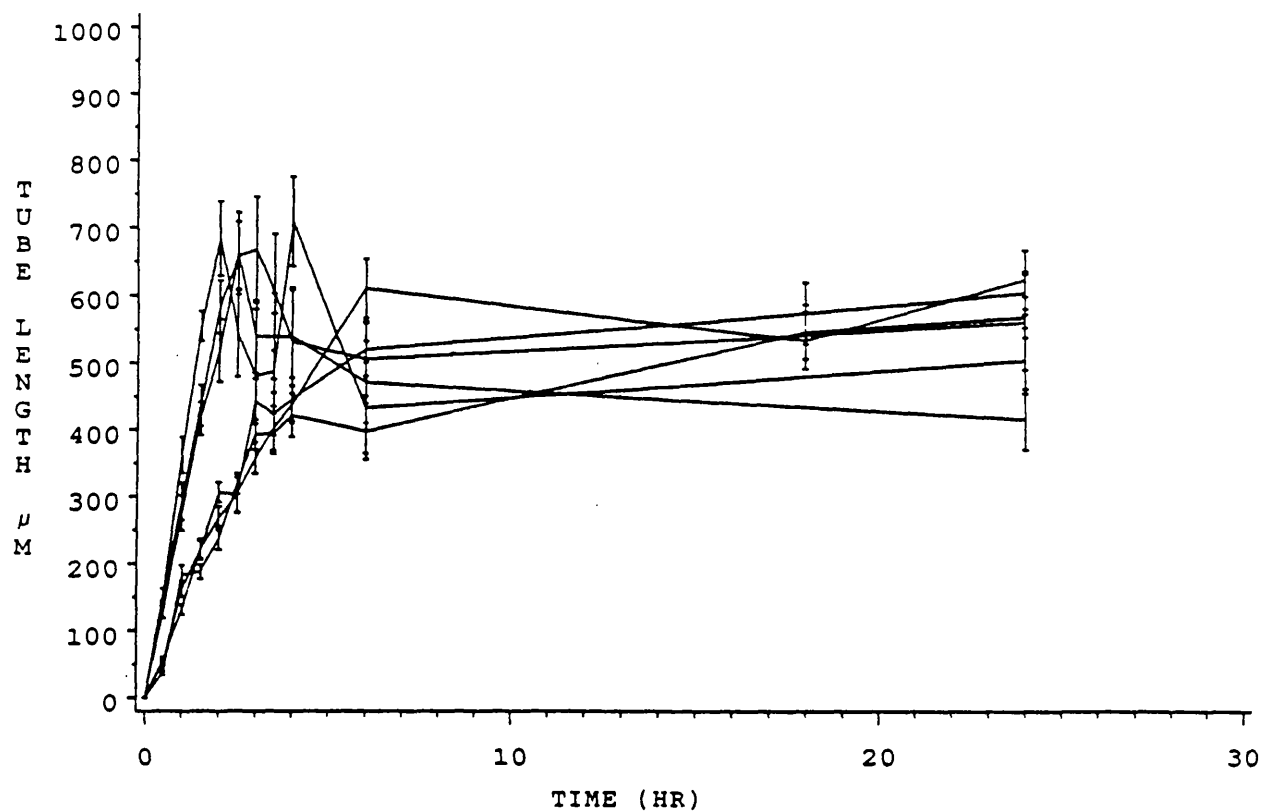


Fig. 3.39 The optimal conditions of the tube growth of pea pollen (cv. Orb; 8 replicates after; the error bars represent standard deviation).

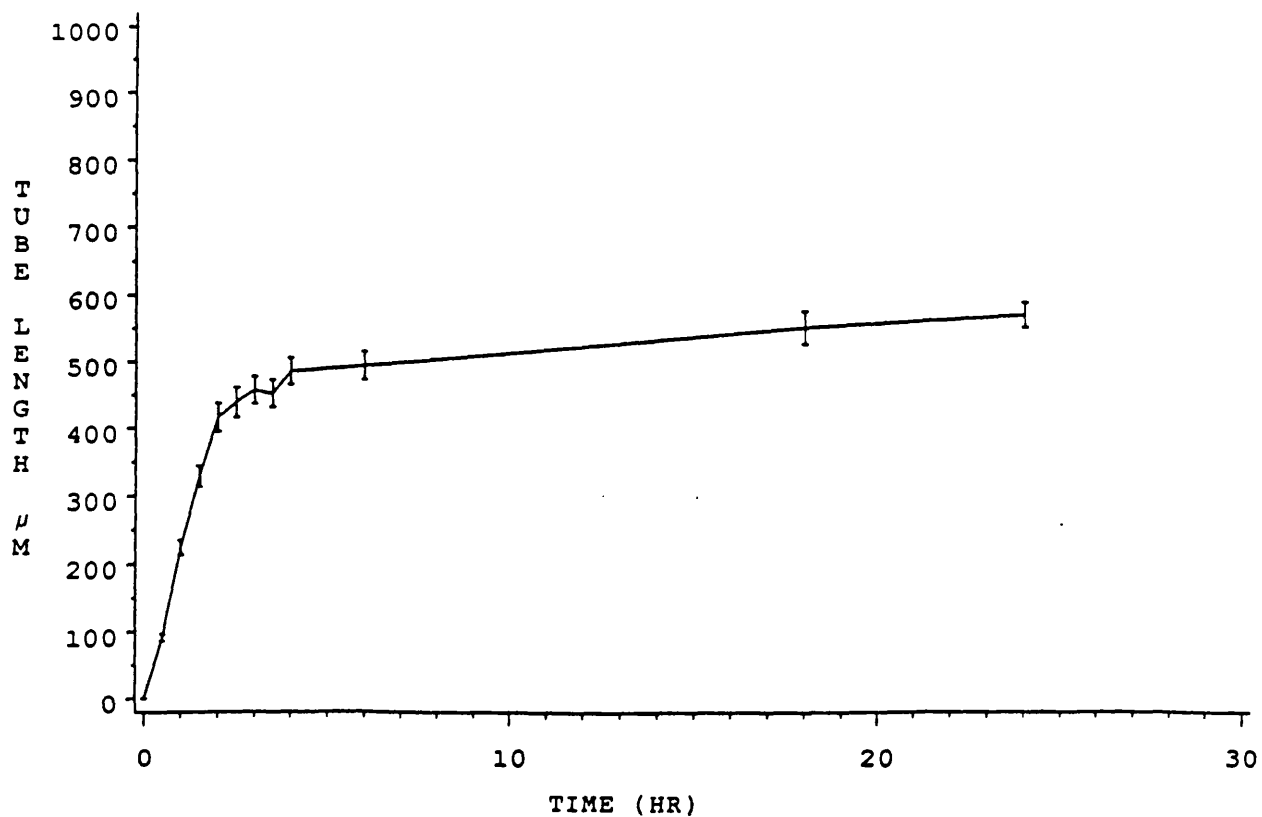


Fig. 3.40 The optimal conditions of the tube growth of pea pollen (cv. Orb; average of the 8 replicates).

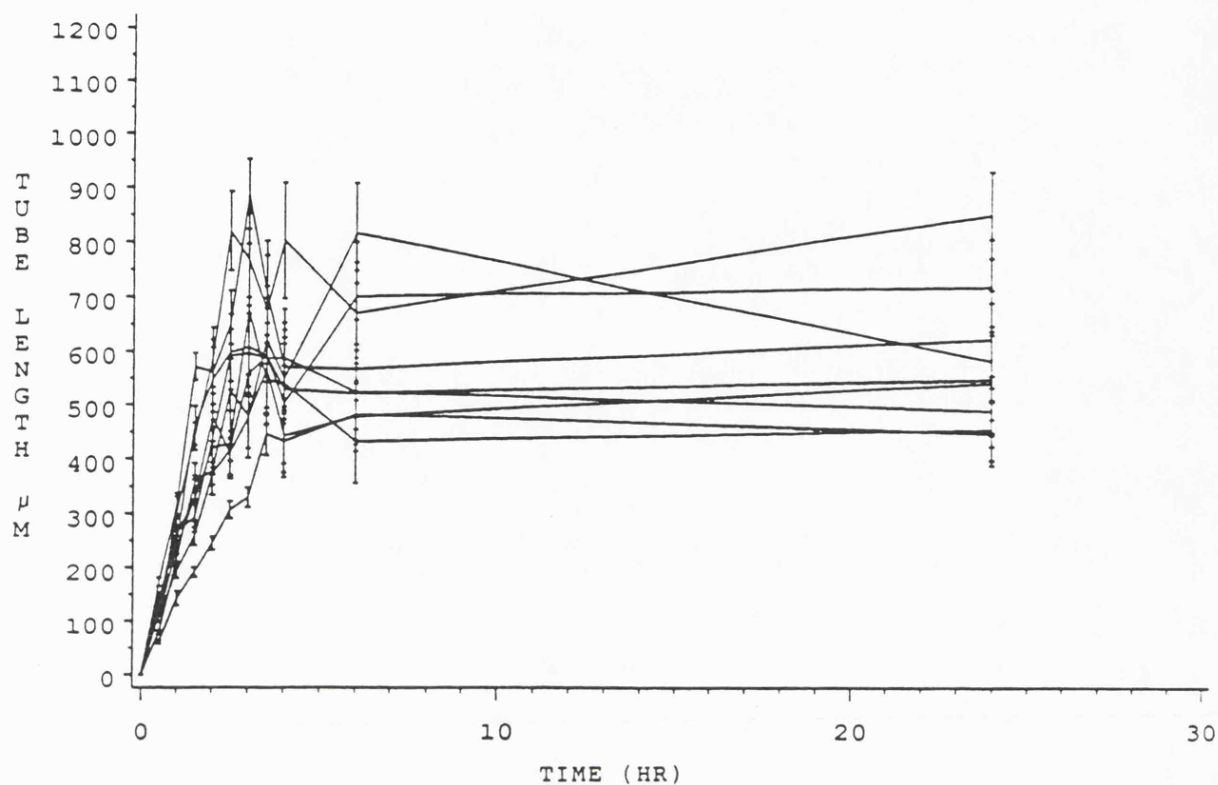


Fig. 3.41 The optimal conditions of the tube growth of pea pollen (cv. Consort; 8 replicates; the error bars represent standard deviation)

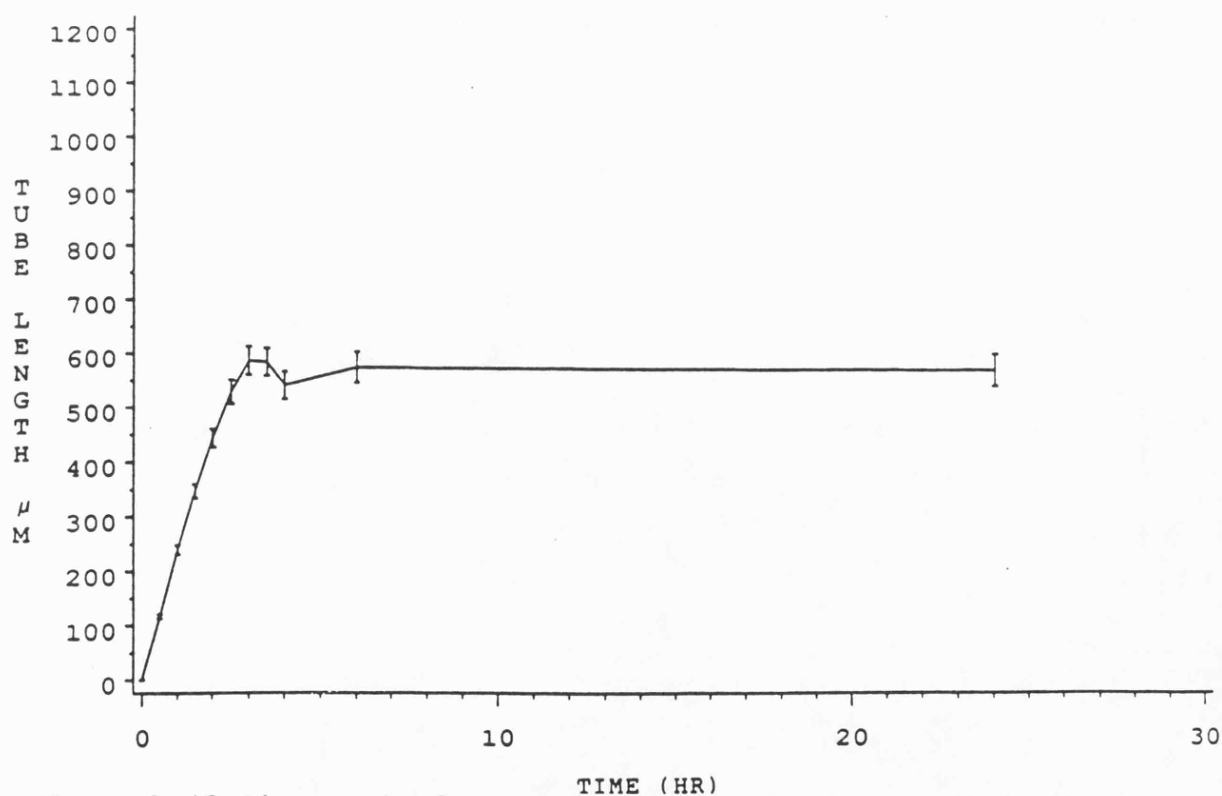


Fig. 3.42 The optimal conditions of the tube growth of pea pollen (cv. Consort; average of the 8 replicates; the error bars represent standard deviation).

placed into culture. Percentage germination rose sharply after this time, reaching a maximum after about an hour; no, or little further, increase was noted after this point. Final percentage germination was high in all replicates, with 68 - 98% and 72 - 96% being observed in Orb (Fig. 3.43) and Consort (Fig. 3.44) respectively.

3.8 DISCUSSION

Of the nine variables investigated during the present study only light was found to have no detectable effect on the performance of pea pollen in culture. These results suggest that the relationship between sucrose concentration and the performance of pollen in culture is comparatively simple for pea. A broad optimal peak of sucrose concentration was observed in both of the cultivars examined, with the greatest tube growth and percentage germination both occurring at about 23% sucrose. In the absence of any data, however, it is not clear whether previous reports of optimal growth occurring between 15 and 32% sucrose (Nair and Singh, 1972; Setia and Malik, 1984) differed from this because of genetic differences between cultivars, differences in culture conditions or through other factors. The role played by boron in the germination of pollen grain is highly complicated (for review see Vasil, 1987) and may alter with the complexity of the medium. Certainly, pea pollen in BM and SM exhibited different responses to changes in boron concentration. The enhancement of tube growth and percentage germination evident at certain concentrations of boric acid in BM was acute, with excess and sub-optimal

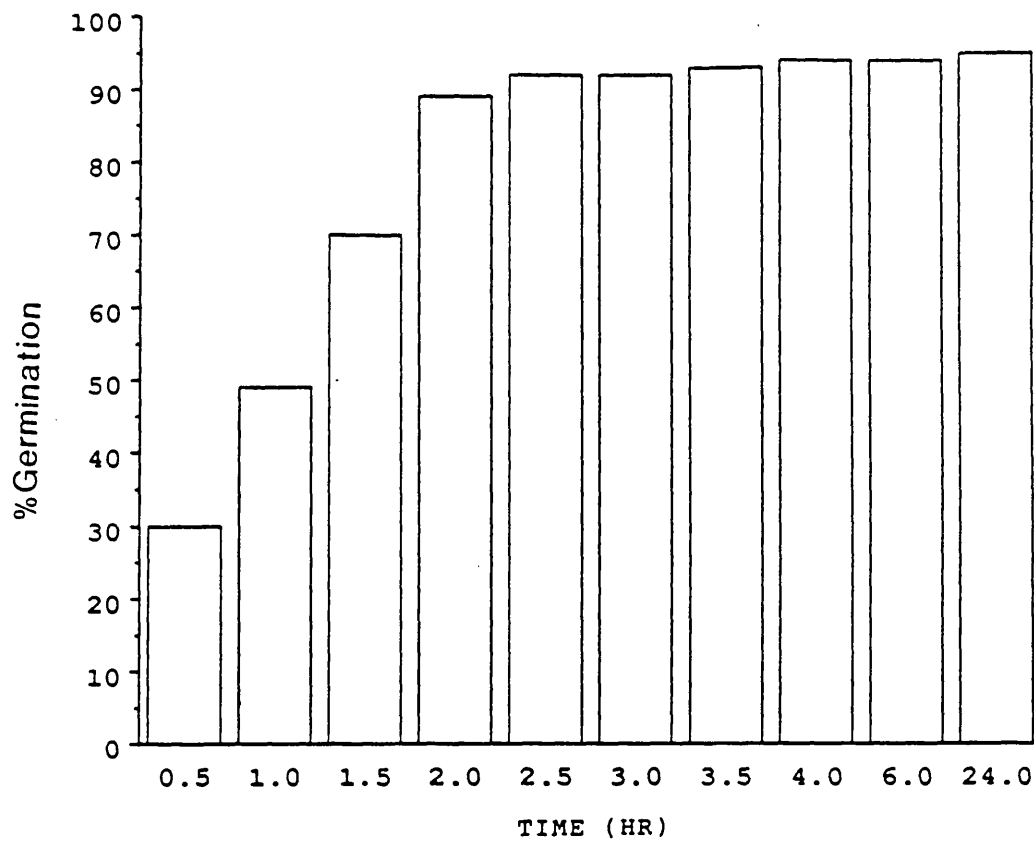


Fig. 3.43 The % germination of the optimal conditions of pea pollen (cv. Orb)

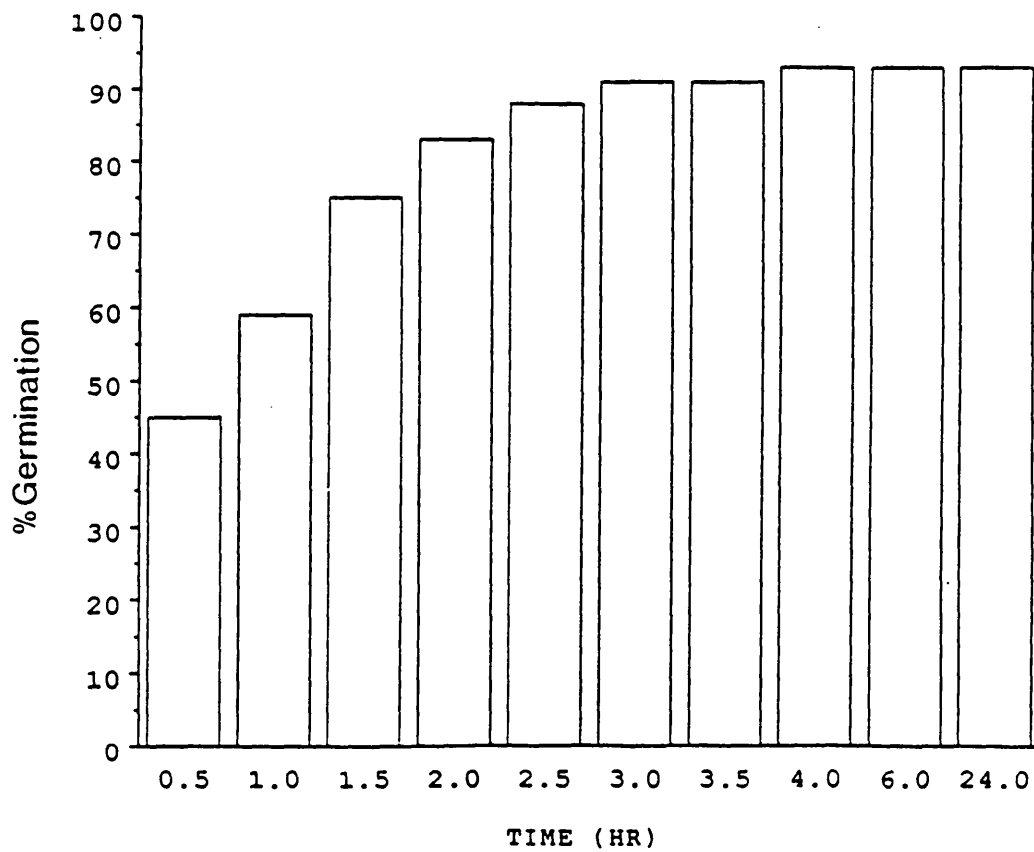


Fig. 3.44 The % germination of the optimal conditions of pea pollen (cv. Consort)

levels both proving inhibitory. This was in contrast to observations in SM, where the lower levels of boric acid (1 - 100 mg/l) produced broadly similar tube growth and percentage germination; This difference may be of some relevance to studies which use simple media to investigate the effects of boric acid in culture and might point towards a dual, or more complex, role of the anion.

In common with many species, the percentage germination and tube growth of pea pollen was greatly improved by the addition of certain mineral salts to the basal germination medium. The two complex media used in the present study (BM and FM) differ greatly in their anion components but are broadly similar in their cation constituents. Since pea pollen performed equally well in both media it can be inferred that whilst the addition of some cations is highly beneficial to germination (viz. Mg^{2+} , Ca^{2+} , K^{+}) the anion component of the medium seems to be largely unimportant.

The pH of the incubation medium had a significant effect on tube growth and on percent germination. Optimal tube growth occurred at pH 6.5 - 6.75, with inhibition of growth above and below this range. A broadly similar percentage germination was achieved in media with a pH in the range of pH 5 - 6.5, but inhibition occurred in media of pH 6.5 and above. These factors combine to suggest a fairly tight pH optimum of around 6.5. Nair and Singh (1972) inferred that the use of buffers to adjust pH rather than NaOH/HCl may affect the germination of pea pollen in culture. In the present study no detectable differences between germination in media adjusted with

Tris HCl and that adjusted with NaOH/HCl were found.

Pea pollen was found to be less sensitive to changes in temperature than had been reported by Setia and Malik (1984). A similar performance was noted in all pollen samples incubated at temperatures between 15 and 25°C, with inhibition of tube growth and percentage germination occurring outside this range. In contrast, a strong enhancement of performance was obtained by continuous shaking of the medium during the incubation period.

Pollen incubated at densities below 35,000 grains/ml appeared not to differ in their percentage germination or on their final tube lengths. At densities above this value, both parameters were inhibited. Competition for nutrients, toxic pollen exudates and/or excess calcium from the pollen and medium may all have contributed towards the inhibition at the higher incubation densities. Furthermore, should excess calcium be a significant inhibitory factor, then different levels of calcium in medium may be required for different incubation densities. However, doubling the calcium component of BM failed to affect germination, suggesting that calcium is not a major inhibitory factor and that calcium levels of BM are not suboptimal for pea pollen.

The physiological state of the pollen donor plants appeared to play a critical role in the germination and tube growth of pea pollen in vitro. Pollen from old, infected and infested plants consistently germinated less well and produced shorter tubes. Pollen taken from indehiscent anthers or from anthers which had dehisced for more than 3 - 4 hr behaved similarly. The cleistogamous

flowers of the cultivated pea can also make identification and collection of sufficient quantities of suitable material problematical. However, since the initiation of germination is highly synchronous, and since tube growth is more or less constant during the first four hours of culture, it is usually possible to distinguish between 'good' and 'poor' pollen batches just 30 minutes into the incubation period. For instance, pollen samples with a mean tube length of more than 130 μm after this time will usually produce final tube lengths of 800 - 1500 μm , whereas those with a mean tube length of 60 μm or less will only rarely produce final tube lengths exceeding 600 μl .

The described system allows the routine germination of large pollen samples with a high percentage germination and good tube growth. In turn, it is hoped that this will allow further progress to be made on pollen-based studies on gametophyte selection and transformation in pea.

3.9 TOBACCO AND PEA FERTILISATION SYSTEM

A major prerequisite for obtaining pollen-mediated transformation is establishing an effective fertilisation system with *in vitro* germinated pollen that has received vector DNA.

3.9.1 In vitro fertilisation

As mentioned in section 3.1, the fertilisation biology of many plants has been studied out *in vivo* and *in vitro* with the purpose of overcoming sexual incompatibility.

The technique of intra-ovarian pollination was achieved and successfully applied in many members of Papaveraceae. Pollen suspensions of five species of poppies were separately placed in a solution of boric acid (100 - 200 mg/l) in sterile double distilled water. After wiping the surface of the ovary with ethanol, two punctures were made on it. With an all-glass 'insulin' syringe the pollen suspension was injected through on the aperture until the ovarian cavity was full. The other served as an outlet for the air. After the operation, the holes were sealed with petroleum jelly. All the five species produced viable seeds which on germination produced plants which flowered as in nature (Kanta and Maheshwari, 1963).

Test tube fertilisation has been achieved in many angiosperm species (e.g. *Papaver somniferum*, *Argemone mexicana*, *Eschscholiza californica*, *Nicotiana rustica* and *N. tabacum*). In this technique, the ovaries were

sterilised, then the ovules were scooped out of the ovary with an aseptic scalpel and sown on simple nutrient agar medium. Pollen taken from anthers was spread around as well as on the surface of the ovules. A double fertilisation and seed development *in vitro* was achieved (Kanta et al., 1962). In another report, test tube fertilisation was achieved by using ovules of *Melandrium album* with pollen grains of several species of Caryophyllaceae family (Zenkteler, 1967; 1970).

Fertilisation has been studied using pistil culture. *Petunia* flower buds were excised before anthesis. The basal portion was sterilised and washed. The petals and stamen were removed aseptically. The pistil was implanted on Nitsch's medium and pollinated with a sterile needle either on the same or the following day. Sections from a 10-day-old culture showed the presence of globular embryo and cellular endosperm in the majority of the ovules. In an ovule from a 2-week-old culture the cotyledon had already been initiated. Three-week-old cultures showed older embryos although in some ovules they were still at the heart-shaped stage. Unequal cotyledons were noticed in a few embryos. About 150 - 200 seeds were produced in each ovary and gave rise ultimately to normal seedlings (Shivanna, 1965). In another report, flowers buds of *Antirrhinum majus* were cultured and about 150 seeds were obtained in contrast to 400 - 500 seeds developed under field conditions (Usha, 1965).

In *petunia*, self incompatibility was overcome by pollinating the whole placentae *in vitro* (Shivanna, 1971). The 2 placentae were slit with a sterilised scalpel almost

to the base of the septa and a piece of cellophane was inserted in the slit. One of the placentae was self-pollinated and the other was cross-pollinated. Within 5 days of pollination, many cultures showed the beginning of seed development on both placentae, and mature seeds were formed in 21 days. In tobacco, whole placentae with intact ovules of *N. tabacum* were pollinated in vitro by dusting with pollen grains of *N. rustica*. Developed ovules were excised from the placentae and transferred to ovule culture. 12 hybrid plants were obtained (Marubashi and Nakajima, 1985). The attempts to hybridize *Nicotiana tabacum* with *Petunia axillaris* was unsuccessful (DeVerna and Collins, 1984); though hybrid plants were not produced, three of the four cross combinations produced plants that were similar in appearance to the source used as the maternal parent.

In vitro pollination of placenta-attached ovules was useful in bypassing unilateral incongruity barriers for several *Nicotiana* interspecific hybrid combinations (*N. tabacum* cv. 'Ky' x *N. amplexicaulis*, 'Ky' x *N. benthamiana*, and 'Ky' x *N. repanda*) (DeVerna et al., 1987). Interspecific hybrids in *Nicotiana* were obtained through in vitro culture of fertilised ovules (Reed and Collins, 1978), by crossing *N. repanda*, *N. stocktii*, and *N. nesophila* with both diploid and tetraploid *N. tabacum*. This technique involved aseptically excising hybrid ovules from ovaries at various time following pollination and placing them on a medium. Seed was obtained only when the wild species were used as the female parent and diploid *N. tabacum* was used as the pollen parent. 10 plants were

produced from 100 cultured ovules of *N. repanda* x *N. tabacum* and 100 plants from 151 cultured ovules of *N. stocktonii* x *N. tabacum* and 8 plants from 136 cultured ovules of *N. nesophila* x *N. tabacum*.

In other reports, pregerminated tobacco pollen was applied on stigma after 10, 30 min, 1, 2, 4 and 6 hr. The pregerminated pollen was separated from the medium by filtration. The highest seed number was obtained after 'pollination' with a 1 hr pollen tube culture (48 seeds). With pollen cultivated longer than 4 hr, no seed formation was observed. The elongation of pollen tubes ceased after 9 - 11 hr when the tube have an average length of 1698 μ m (Balatkova et al., 1977).

In general, the plant condition affects the pollen viability and subsequently the obtained seeds (Young and Stanton, 1990); pollen of *Raphanus raphanistrum* produced under low nutrient conditions (no fertiliser) sired fewer seeds than pollen produced under better conditions (fertilised with Hoagland's solution, two to three times weekly) when the two types were applied onto a stigmas together.

3.9.2 Fertilisation with in vitro germinated tobacco pollen

3.9.2.1 Pollen germinated in liquid media

Pollen of tobacco was germinated in vitro (see Chapter 2; section 2.5.1), and then transferred to different stigmata to find out which stigmata produces the highest number of seeds and subsequently to be used for transformation experiments. The stigmata were:

- i- An untreated stigma.
- ii- A stabbed stigma.
- iii- A stigma which had been cut at the tip.
- iv- The cut edge of a sliced stigma and/or style.

Stigmata pollinated with pollen germinated in liquid media produced between 18 and 87 seeds per ovary. Control ovaries fertilised normally yielded between, 1800 and 2500 seeds per ovary, so artificial pollination produced between 1% and 4% normal seed set. This figure was improved by pollinating twice at 6 hr intervals. Similar fertilisation results were obtained when liquid germinated pollen was applied to stabbed stigmas, and cut stigmas (Table 3.1; See Appendix 1). No seeds were obtained when pre-germinated pollen was applied to sliced stigmas or styles.

3.9.2.2 Tobacco fertilisation occurred from pre-germinated pollen

Tobacco pollen was germinated in SM for 2.5 hr and 90% germination was observed. The germinated pollen was separated into two samples. 1 ml distilled water was added to the first sample; this treatment made germinated pollen inviable through burstage, but had no visible affect on ungerminated grains. The pollen was spun down (6500 rpm) for 30 - 45 sec and the supernatant decanted. No distilled water was added to the control sample.

Stigmata were pollinated with either the sample containing osmotically burst pollen and (10%) ungerminated pollen or the untreated control sample. It was realised that any seed set obtained from pollen incubated in liquid

Artificial pollination	No. of seeds	Percentage in comparison to the control
Single pollination	18	1
	26	1.3
	36	1.8
	87	4.3
Double pollination	187	9.3
	215	10.7
Stabbed stigmas	68	3.4
	112	5.6
Cut stigmas	101	5
	151	7.5
Control	Approx. 2000	

Table 3.1 The effect of artificial pollination on tobacco fertilisation.

germination medium may have been from the small proportion of ungerminated grains rather than the germinated pollen. If this was true, then it would greatly reduce the probability of obtaining transformation through the co-incubation of plasmid and germinating pollen.

None of the ovaries pollinated with osmotically burst samples set seed. However, 50% of the ovaries pollinated with the untreated sample set some seeds (32, 46 and 69 seeds).

These results suggest that the 10% of the pollen which did not germinate after 2.5 hr also failed to germinate subsequently. By implication, therefore, these data suggest that it was the pre-germinated pollen which fertilised the ovaries and gave rise to seed set in pollinations from liquid media.

3.9.2.3 Fertilisation from static and shaken pollen culture

Pollen from freshly dehisced anthers from 3 flowers (15 anthers) were added to 8 ml of SM adjusted to pH 6.75. After mixing, the sample was divided into two equal halves. One of these was shaken and the other left to stand. Every 30 min until 4 hr, 0.5 ml from each experiment was taken, filtered and the pollen was applied to emasculated flowers. Percentage germination and number of seeds set were recorded. The experiment was performed twice for each treatment. The number of seeds obtained from pollen germinated in static cultures was high in the first two hours, then the number reduced after 3 - 4 hr (Table 3.2). In the shaken treatment the number of seeds

Time (hr)	No. of seeds from shaken culture	No. of seeds from static culture	Percentage of pollen germination at time of pollination
0.5	890	1141	41.3
1	613	931	74.5
1.5	510	550	79.9
2	263	420	91.0
2.5	200	590	91.2
3	251	282	92.6
3.5	209	244	92.6
4	0	90	94.6

Table 3.2 The effect of shaken and static tobacco pollen culture on fertilisation.

was a little lower and no seeds were obtained after 4 hr (Table 3.2).

The percentage germination was low after 0.5 hr, then increased between 1 - 4 hr and then was no difference between fertilisation by shaken and static cultured pollen.

After 0.5 hr the percentage germination was low and most of the tubes were short and this gave a chance for a higher number of pollen grains to germinate onto the stigma or for the pollen with short tubes to penetrate. After 2 - 4 hr the number of seeds was reduced because most of the pollen tubes were long and the germination energy reserves had probably been almost consumed, and even if the tube penetrated it could be difficult for it to reach the ovule.

This data suggest that DNA uptake into pollen would have to be active between 0.5 and 2.5 hr to achieve a reasonable level of seed set in tobacco.

3.9.2.4 Injection into ovaries

Pollen was germinated in SM. A Hamilton syringe (100 μ l) was used to inject the pollen suspension into the ovary locules. Many apertures were used since it has plurilocular ovary (2 fused carpels). The apertures were sealed with paraffin wax after injection of the pollen suspension. 16 ovaries were treated. Seeds were collected after 8 weeks. Over 60% of ovaries injected with germinated pollen produced various number of seeds (Table 3.3). 6 ovaries failed to produce seeds.

Number of seeds
103
36
81
39
77
201
43
91
55

Table 3.3 Number of seeds obtained from ovaries of tobacco injected with pre-germinated pollen.

3.9.3 Pea

3.9.3.1 Pea pollen germinated in different cultures and subsequently applied on pea stigmata or injected into pea ovaries

Pea pollen germinated in liquid medium, 30 seeds were obtained from the 70 pods (0.4 seeds per pod; Table 3.4) derived from flower pollinated with pollen pre-germinated in BM. This compares with 89 seeds from 22 pods (4.04 seeds per pod) obtained in the control plants. Therefore, approximately 10% of natural set was obtained. Fertilisation with pea pollen germinated on cellophane (see Chapter 2; section 2.5.2) and collected in liquid media was rather poor; 10 seeds were obtained from 130 pods (0.08 seeds per pod; Table 3.4). This compares badly with the 45% - 65% obtained naturally. Pea pollen germinated in semisolid media (see Chapter 2; section 2.5.3) resulted in 20 seeds from 51 pods (0.4 seeds per pod; Table 3.4). Injection of 50 pea ovaries with pollen pre-germinated in BM failed to produce seeds.

Seed obtained from in vitro fertilisation experiment were all grown to maturity under green-house conditions.

For pea there have been no reports regarding in vitro fertilisation and so in this study, in order to establish the reason for the low success rate in which ten ovules out of 130 were fertilised and produced a seed, the natural system was re-examined. Naturally pollinated stigmata were stained with aniline blue and examined for pollen tube growth and penetration. Three points were

noted:

i- The region of the stigmatic receptive to pollen tube penetration was very small and restricted to the tip (Fig. 3.45).

ii- Over the rest of the stigma and style, pollen

germination media	No. of plants	No. of pollinated stigmas	No. of pods	No of seeds
Liquid media	22	109	70	30
Cellophane method	39	198	130	10
Semi-solid media	15	97	51	20

Table 3.4 Effect of different media on pea fertilisation

concentration of pollen and by pollinating twice.

Using these high concentrations of pollen 100% of the ovules set seeds suggesting that a further improvement in pollen density may help to optimise artificial seed set.

3.10 SUMMARY

This chapter described the optimum conditions for pollen germination and fertilisation in pea and tobacco. Regarding the dehiscence time, it appeared that there was no specific time for tobacco anther dehiscence. For pea, the anthers started dehiscence after 11 am (or approximately 6 hr after sunrise). SM (15% sucrose, 100 - 150 mg/l boric acid, pH 5.5 - 6.75, 15 - 30°C and 6000 - 27000 grains/ml) was the medium of choice for tobacco; it gave the greatest tube length and percentage germination. For pea, BM media (33% sucrose for both Oib and Consort, 100 mg/l, pH 5.5) shaken at 15 - 25 °C with 25000 - 35000 grains/ml gave optimum germination and tube growth.

noted:

i- The region of the stigmatic receptive to pollen tube penetration was very small and restricted to the tip (Fig. 3.45).

ii- Over the rest of the stigma and style, pollen germination was actually repressed.

iii- pollen density in the natural system was about 100 times greater than that used in the artificial system.

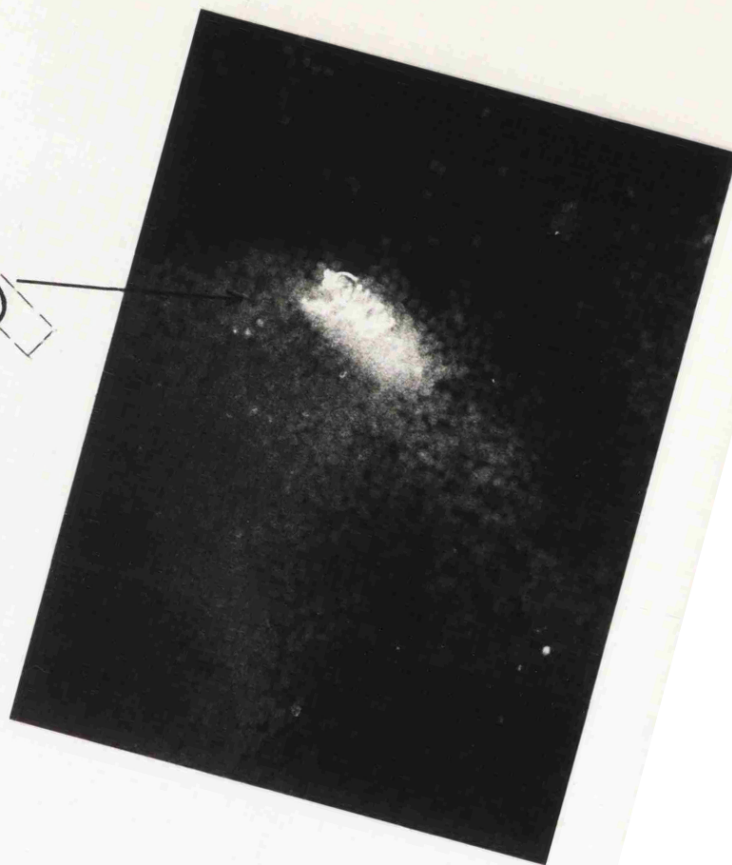
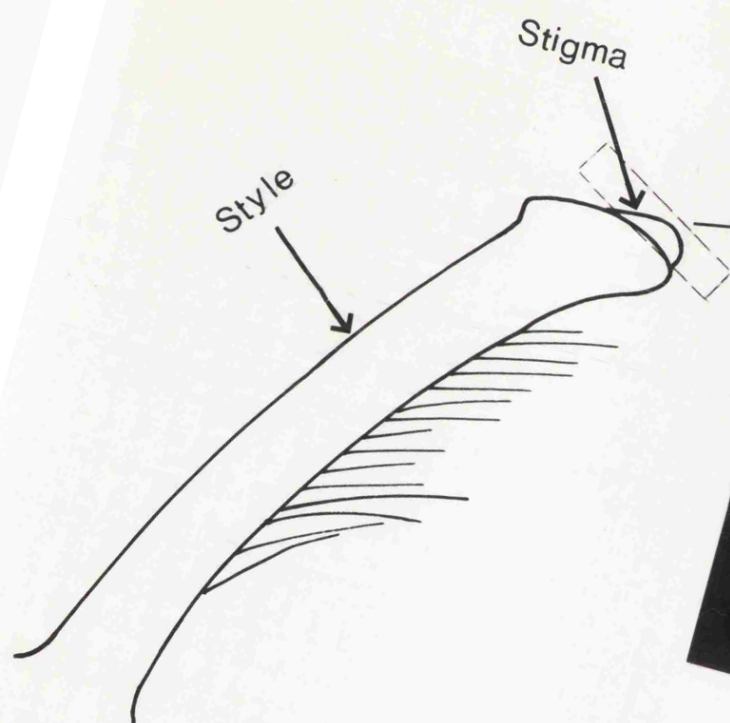
It seemed probable from this work that pollen density may have been the major cause of the low seed set obtained via 'artificial' fertilisation. In order to test this theory, the experiment was repeated using 2-3X the concentration of pollen and by pollinating twice.

Using these high concentrations of pollen 10% of the ovules set seeds suggesting that a further improvement in pollen density may help to optimise artificial seed set.

3.10 SUMMARY

This chapter described the optimum conditions for pollen germination and fertilisation in pea and tobacco. Regarding the dehiscence time, it appeared that there was no specific time for tobacco anther dehiscence. For pea, the anthers started dehiscence after 11 am (or approximately 6 hr after sunshine). SM (15% sucrose, 100 - 150 mg/l boric acid, pH 6.5 - 6.75, 15 - 30°C and 6000 - 27000 grains/ml) was the medium of choice for tobacco; it gave the greatest tube length and percentage germination. For pea, BM media (23% sucrose for both Orb and Consort, 100 mg/l, pH 6.5) shaken at 15 - 25 °C with 25000 - 35000 grains/ml gave optimum germination and tube growth.

Fig. 3.45 The region of pea stigma receptive to pollen tube penetration (x 65).



Many techniques were used by other research workers for in vitro fertilisation and these techniques gave different numbers of seeds depending on the species. Fertilisation of petunia by pollination of the pistil in vitro gave between 150 - 200 seeds whereas for *Anthriscum*, 150 seeds were obtained (Shivanna, 1965). In vitro pollination of placental-attached ovules of *Nicotiana* gave 118 plants out of 296 crosses (Reed and Collins, 1978). By applying pregerminated pollen for 1 hr on tobacco stigmas, 48 seeds were obtained (Balatok et al., 1977). In comparison to these results with the present study showed that after applying pregerminated pollen to tobacco stigmas, the highest seed set was 215. 187 seeds were obtained with double fertilisation; 112 seeds with stabbed stigma; 151 seeds with cut stigma and 87 seeds with a single fertilisation. For pea, 0.4 seeds per pod were obtained after applying pre-germinated pollen in BM on stigmas compared to a control value of 4.04 seeds per pod. The number of seed obtained in this study is satisfactory for pea and tobacco in comparison with the previous work and the reason for the low numbers of seed set in general is probably that the pregerminated pollen grain with a longer tube growth did not always penetrate or in the case of penetration did not always reach the ovules and also the amount of pollen that was placed on the stigmas was probably effectively less than in the control.

Appendix 1

t-tests (two-tail) for the experiments described in chapter 3.

3.4.1	t	P	Signification
BM - FM	2.969	0.01	Not sig.
BM - SM	4.596	0.01	Not sig.
FM - SM	1.626	0.01	Not sig.

3.4.2	t	P	Signification
10% - 15%	1.414	0.01	Not sig.
10% - 20%	12.020	0.01	Not sig.
15% - 20%	13.435	0.01	Sig.

3.4.3	t	P	Signification
10 - 50	2.121	0.01	Not sig.
10 - 100	5.656	0.01	Not sig.
50 - 100	7.778	0.01	Not sig.

3.4.4	t	P	Signification
6.75 - 7	4.240	0.01	Not sig.

3.4.5	t	P	Signification
20 - 25	6.363	0.01	Not sig.
20 - 30	38.183	0.01	Sig.
25 - 30	24.748	0.01	Sig.

3.4.6	t	P	Signification
6000 - 27000	4.949	0.01	Not sig.

3.4.7	t	P	Signification
Shaken-static	2.828	0.01	Not sig.

3.4.8	t	P	Signification
Genotypes	3.674	0.01	Not sig.

3.7.1	t	P	Signification
Consort-Orb	1.767	0.01	Not sig.

3.7.2 (SM)	t	P	Signification
10% - 50%	1.414	0.01	Not sig.
10% - 100%	1.767	0.01	Not sig.
50% - 100%	3.181	0.01	Not sig.

3.7.2 (BM)	t	P	Signification
0 - 100	5.303	0.01	Not sig.
0 - 200	1.767	0.01	Not sig.
100 - 200	3.535	0.01	Not sig.

3.7.3	t	P	Signification
BM - FM	10.606	0.01	Not sig.

3.7.4	t	P	Signification
6.50 - 6.75	4.949	0.01	Not sig.
6.50 - 7	1.141	0.01	Not sig.
6.75 - 7	6.363	0.01	Not sig.

3.7.4	t	P	Signification
NaOH - Tris	2.828	0.01	Not sig.

3.7.5	t	P	Signification
15 - 20	2.828	0.01	Not sig.
15 - 25	3.535	0.01	Not sig.
20 - 25	1.414	0.01	Not sig

3.7.6	t	P	Signification
Shaken-static	13.435	0.01	Sig.

3.7.7	t	P	Signification
2500 - 3500	7.071	0.01	Not sig.

3.7.7 (Orb)	t	P	Signification
5 - 10	4.242	0.01	Not sig.
5 - 20	6.363	0.01	Not sig.
10 - 20	10.606	0.01	Not sig.

3.7.7(Consort)	t	P	Signification
5 - 10	1.060	0.01	Not sig.

5 - 20	3.889	0.01	Not sig.
10 - 20	2.828	0.01	Not sig.

3.9.2.1	t	P	Signification
Single - double	112.606	0.01	Sig.
Single - stabbed	34.117	0.01	Sig.
Single - cut	59.573	0.01	Sig.
Double - stabbed	78.488	0.01	Sig.
Double - cut	53.033	0.01	Sig.
Stabbed - cut	25.455	0.01	Sig.

CHAPTER **4**

4.0 DNA UPTAKE INTO POLLEN IN VITRO

Introduction

The importance of the pollen tube as a carrier of two sperms to the ovules and therefore offering an ideal target for transformation has been recognised for many years. In spite of the hard work which has been performed to try and transform pollen since the early seventies (Hess et al., 1975; Hess, 1979; Hess, 1981; Hess and Dressler, 1984; De Wet et al., 1985; Hepher et al., 1985; Booy et al., 1989) it is still far from being a routine technology. In this chapter it is appropriate to assess the possibility of introducing foreign DNA into pollen grains and pollen tubes and to detect the presence of labelled DNA inside or outside the pollen tube. Firstly,

information about direct gene transfer techniques to plant cells and pollen grains/tubes which have been used is provided in section 4.1 and the advantages and disadvantages of each method is discussed.

4.1 GENE TRANSFER TO PLANT CELLS IN VITRO

The genetic transformation of plants was first carried out using *A. tumefaciens* and *A. rhizogenes* (see Chapter 1; section 1.2), and most of the transgenic plants to date have been produced through the use of the *Agrobacterium* system. Monocots, which include the economically valuable cereal crops, appear not to be susceptible to transformation by *Agrobacteria* in the same way as dicots. The reason for this is not clear, but it may be because bacteria do not attach to cereal cell walls, or because hormonal response are different in cereals, or because specific wound substances are absent, or present at too low concentrations, at wound sites to induce *vir* region transcription on the Ti plasmid in order to stimulate the transfer process. Wounding in monocots generally leads to the death of the wound-adjacent cells (Lindsey and Jones, 1989c; Potrykus, 1990). Additionally, there are many problems associated with the tissue culture of monocots in general. One of the problems is plant regeneration. As mentioned in Chapter 1, the most successful transformation events occur in single de-differentiated cells. The success of regeneration from callus, organ explants or single cells especially in monocot or woody species is very limited for unknown reasons. If regeneration is possible, some problems could

occur, such as somaclonal variation. Also, the karyotype could be affected in regenerated plant (either numerical changes; ployploidy or aneuploidy); this may be direct result of auxin or cytokinin in the medium. Thus, it may be appropriate to consider exploiting transformation methods which avoid regeneration from dedifferentiated cell types.

Introducing DNA into cells is inhibited by the existence of the cell wall barrier; but removal of the cell wall renders the resulting protoplasts amenable to various techniques of genetic transformation (PEG, electroporation, and DNA-encapsulating liposomes). On the other hand, removing the cell wall makes the protoplasts fragile. The osmotic problem arising from the existence of a large central vacuole and a relatively weak cytoplasmic envelope in many cells has resulted in the failure of many previous experiments especially with microinjection methods; this is the reason why the efficiency of microinjection for transformation is higher in animal cells than in plant cells (Steinbiss and Stable, 1983). Introducing DNA into cells could be affected by nuclease activity which degrades the DNA either completely as in the case of incubation of cells with DNA or partly when the cells are incubated with liposome-encapsulated DNA. The release of nuclease activity is one of the major problems in pollen transformation which occurs during pollen germination (Matousek and Tupy, 1983; Negrutiu et al., 1986; Westhuizen et al., 1987) and especially when the anthers are squashed. Even without anther tissue, nuclease activities can be detected. This is because the

nucleases can be hidden in the cavities of the exine or between exine and intine (Hess, 1987); a washing procedure of 5 or 10 min before the addition of DNA to the pollen, or coating of pollen by Denhardt's solution, or varying the pH (4 - 6), or the addition of EDTA or phosphate did not remove much of the nuclease activity; it is thought that the nuclease activity is released gradually into the media (Negrutiu et al., 1986; Hess, 1987; Booy et al., 1989). Nucleases may well be present on the stigma and also in the stylar channel. These organs have not only the function of allowing the pollen tube to enter the ovary, but also of protecting the ovary from alien agents (pathogens) and to nourish the pollen tube during its passage through the style (Heberla-Bors et al., 1990)

Since the late 1970s, several physical methods for direct gene transfer into plant cells have been developed and have resulted either in a transient expression or stable transformation. These methods can be classified in two groups; those which normally require protoplasts and do not work with other cells (PEG, electroporation and liposomes), and methods which do not necessarily require protoplasts and theoretically work with other types of cells (microinjection, microprojectiles). Transformation has been achieved in a variety of species (Table 4.1; Gasser and Fraley, 1989). Transformation of plants was facilitated in the late 1970s by the discovery of plasmids which could be isolated easily from bacteria, and also the development of sensitive and accurate techniques such as gel electrophoresis and DNA hybridisation by Southern blotting (Southern, 1975). Because plasmids have been

Plant species	Method
Herbaceous dicot	
Petunia	At
Tomato	At
Potato	At
Tobacco	At, FP, PG
Arabidopsis	At
Lettuce	At
Sunflower	At
Oilseed rape	At, MI
Flax	At
Cotton	At
Sugarbeet	At
Celery	At
Soybean	At
Alfalfa	At
Medicago varia	At
Lotus	At
Vigna aconitifolia	FP
Cucumber	Ar
Carrot	Ar
Cauliflower	Ar
Horseradish	Ar
Morning glory	Ar
Woody dicot	
Poplar	At
Walnut	At
Apple	At
Monocot	
Asparagus	At
Rice	FP
Corn	FP
Orchid	FP
Rye	IR

Table 4.1 Species for which the production of transgenic Plants have been reported. Abbreviation:
 At, *Agrobacterium tumefaciens*.
 Ar, *Agrobacterium rizogenes*.
 FP, free DNA introduction into protoplasts.
 PG, particle gun.
 Mi, microinjection.
 IR, injection of reproductive organ.
 (from: Gasser and Fraley, 1989).

used for general direct gene transfer, a good deal of effort has gone into development of selectable marker genes (antibiotic resistance genes) and screenable marker genes (Table 4.2), engineered to be expressed in plant cells and each marker gene may have advantage for certain plant species. In the development of a useful selectable marker, there are several considerations. Foremost, the selective agent must be inhibitory to the growth of plant cells. The presence of dead and dying cells can be very inhibitory to adjacent living cells and this can be a problem. Screenable marker genes are often included on many transformation vectors as even under ideal conditions for transformation, in certain plant species many non-transformants "escape" the selection. So, an independent means of identifying the transformation is desirable. It is very important to have a simple assay for rapid verification of expression of the marker in a regenerated plant. The majority of these selection marker constructs used in the present study have already been tested via *Agrobacterium* transformation and were known to be expressed efficiently in transformed cells.

An ideal transformation vector for direct gene transfer should be of low molecular weight, capable of amplification to a high copy number and easy to isolate, it should carry markers allowing its selection within *E. coli*, and have single restriction sites for a number of restriction endonucleases and also it should have the ability to confer a readily selectable phenotypic trait on host cells. Many small intermediate vectors or binary vectors have been used successfully for direct gene

Selectable markers		
Marker gene	Enzyme encoded	Resistance conferred
NPT II	neomycin phosphotransferase	kanamycin neomycin G418 paromomycin
HPT	hygromycin phosphotransferase	hygromycin
DHFR	dihydrofolate reductase	methotrexate
BLE	bleomycin	bleomycin
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase	glyphosate
Screenable markers		
CAT	chloramphenicol acetyl transferase	
Nos	nopaline synthase	
Ocs	octopine synthase	
GUS	β -glucuronidase	
Lac Z	β -galactosidase	

Table 4.2 Selectable and screenable markers in plant transformation vector (from Lindsey and Jones, 1989c; Draper et al., 1988; Klee and Roger, 1989).

transfer (Draper et al., 1986, 1988; Klee and Roger, 1989; Gasser and Fraley, 1989; Lindsey and Jones 1989c). Such vectors were used in experiments described in this thesis.

4.2 METHODS FOR DNA UPTAKE INTO PLANT CELLS

4.2.1 PEG (polyethylene glycol)

The most widely used of methods of cell fusion utilises polyethylene glycol (m.w 1500, 4000, 6000). PEG is an extremely hydrophilic molecule and it is able to remove much of the 'free water' within a solution; 'free water' being those molecules able to interact with charged (usually ionic) molecules soluble in water. PEG seems to minimise charge repulsion effects, thus cell membranes can come into close contact to allow fusion of lipid bilayers and subsequently, on dilution, cell fusion. Polyethylene glycol has also been shown to be a strong stimulator of endocytosis in plant protoplasts, enabling the uptake of large particulate matter such as whole chloroplasts, liposomes, or bacteria (Draper et al., 1988), but still the precise mode action of PEG is unknown. It appears to disrupt the stability of the plasmamembrane structure, perhaps by acting as a bridging molecule between phospholipid or protein components (Lindsey et al., 1988). PEG has been used successfully to stably transform protoplasts. Between 11% to 40% PEG was used in most of the transformation experiments (Uchimiya et al., 1987; Ballas et al., 1987; Damm et al., 1989). Many species of Gramineae have been transformed by PEG which represents an alternative to *Agrobacterium*-mediated vector delivery because Gramineae cells are not susceptible to

Agrobacterium infection (Chen et al., 1987). *Triticum monococcum* protoplasts were transformed (Lörz et al., 1985), by introducing pPBL1103-4 carrying an NPT-II gene into the protoplasts in presence of 40% PEG 1500 (w/v). Rice (*Oryza sativa*) protoplasts also were transformed by PEG (Uchimiya et al., 1987); the protoplasts were incubated with pCT2T3 carrying an aminoglycoside phosphotransferase II (APH(3')II) gene, with 40% PEG 6000. Colonies capable of proliferating in medium containing kanamycin were selected. A method thought to be applicable for transforming all types of cereal protoplasts was reported (Junker et al., 1987). Maize, rice, and rye protoplasts were transformed by applying PEG in presence of several circular plasmid DNA containing kanamycin resistance genes. The activity of neomycin phosphotransferase was traced 2 days after transformation.

Tobacco protoplasts have been a model for most genetic transformation experiments using PEG. In early studies (Krens et al., 1982), the protoplasts were incubated with pTiAch5 DNA and PEG 6000 (40% w/v) and hormone autotrophic calli selected. The CAT gene (from pCaMVCAT) was used as a marker to detect transformed petunia and carrot protoplasts (Ballas et al., 1987), following PEG (1500, 6000) mediated plasmid uptake. Transformation frequency was dependent to some extent, on both the concentration of PEG and amount of vector DNA (Draper et al., 1988). The highest transformation frequency (about 19%) was observed with *Petunia* protoplasts (11% PEG for 30 min) and the frequency increased to 40%, when the transformation was performed in

the presence of 20 mM CaCl_2 (Ballas et al., 1987); this frequency compared with 20% for *Arabidopsis thaliana* (Damm et al., 1989), 13% for sugarcane protoplasts (Chen et al., 1987), 2 - 3% for *Triticum monococcum* (Lörz et al., 1985), and 0.02% for *Oryza sativa* (Uchimiya et al., 1987). Marker gene activity (e.g. CAT) was detected as early as 5 hr after transformation (Ballas et al., 1987). Transgenic *Arabidopsis* plants resistant to hygromycin B have been regenerated from mesophyll protoplasts which were treated with 20% PEG in presence of plasmid pGL2 containing a hygromycin phosphotransferase gene (Damm et al., 1989). Colonies were analysed by Southern blot hybridisation demonstrating that the foreign gene was stably integrated into plant chromosomes. Sugarcane protoplasts were transformed to kanamycin resistance after plasmid pABDI was added, in the presence of PEG 6000 (40% w/v) for 20 min (Chen et al., 1987). Incubation of cells with PEG causes little cell death (Ballas et al., 1987).

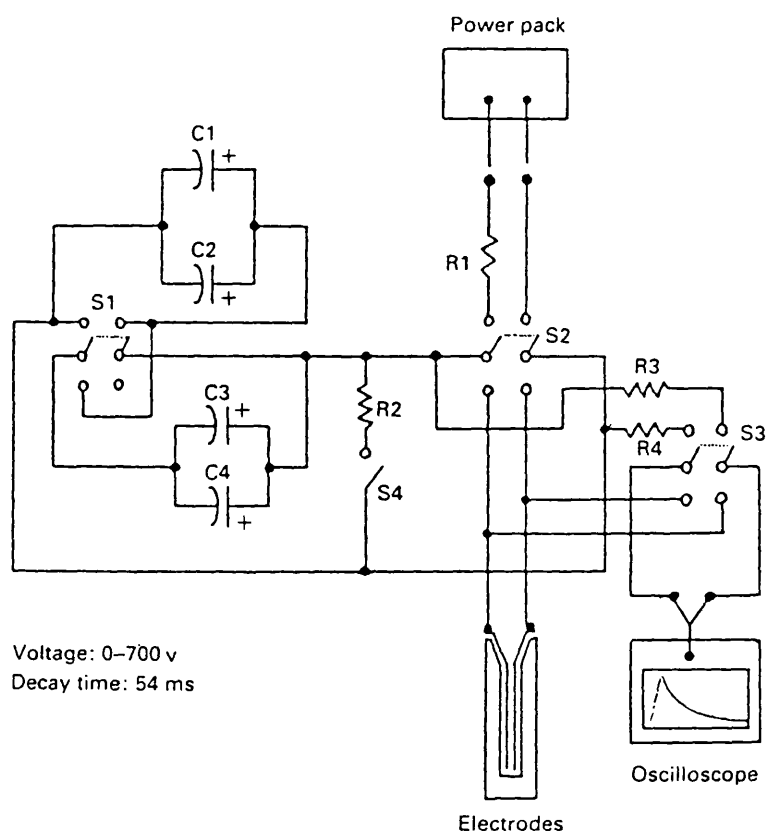
4.2.2 Calcium phosphate precipitation

Transformation was achieved by calcium phosphate co-precipitation of plasmid DNA (Hain et al., 1985). Tobacco protoplasts were incubated with pLGVneo2103, the DNA fragments were precipitated with calcium phosphate. Endocytosis was achieved in presence of polyvinyl alcohol and high pH treatment. Shoots and plants were regenerated from resistant calli.

4.2.3 Electroporation

Electroporation has been used widely because it is a convenient method for transformation both for its simplicity (Fig. 4.1), and the reasonable rate of transformation that can be achieved. Electroporation, as the process has been named, is thought to produce transient pores in the plasma membrane (Zimmerman et al., 1981), through which macromolecules, such as DNA can move into the cell. These pores appear at least 30 nm in diameter and persist for several minutes after the pulse (Okada et al., 1986; Lindsey and Jones, 1989d). Field strength (voltage gradient) and pulse duration (or decay time) are the two main variables affecting the permeabilisation of cell membranes by electroporation. Electroporation-mediated gene transfer has now been developed for several plant protoplast systems. Indeed, within the last few years there have been several reports describing its successful application (e.g. Shillito et al. 1985; Fromm et al., 1985, 1986; Hauptmann et al., 1987; Orad et al., 1989; Lindsey and Jones 1989d; Dimitrov and Sowers, 1990). These holes permit transient expression of foreign genes and production of stable transformation in several species (tobacco, maize, tomato and sugarbeet). Electroporation has been used to achieve a high frequency of DNA delivery in mammalian system genomes and cloned genes have been introduced into mammalian cells (Neumann et al., 1982; Wong and Neumann et al., 1982; Potter et al., 1984; Sowers and Lieber 1986; Dimitrov and Sowers 1990). Fluorescence was observed

Fig. 4.1 Circuit diagram of a low voltage, exponential decay type of electroporator (from Draper et al., 1988).



after electroporation (a single pulse; 0.7 kV, 1.2 ms) of human erythrocyte ghosts loaded with FITC-dextran (mol. mass=10 KDa). Introduction of plasmid DNA (YEpl3) into yeast cells (*Saccharomyces cerevisiae*), by electrical field pulses have been reported (Hashimoto et al., 1985).

Recovery and viability of protoplasts after electroporation decreased with increasing voltage (Hauptmann et al., 1987). Seguin and Lalone (1988), reported that more than 70% of the protoplasts remained viable 2 hr after electroporation; with more pronounced cell death observed at 24 hr after electroporation. For tobacco, the number of living protoplasts after electroporation and incubation was reduced to 40% and 27% of those counted before electroporation (Nishiguchi et al., 1986). The viability of breadwheat protoplasts without electroporation was over 90% as judged by FDA staining. Two hours after electroporation viability dropped from 94% to 63%, 62% and 29% at the 100, 150 and 200 volt setting (Orad et al., 1989). The examination of structural changes by interference phase contrast microscopy of electroporated and non-electroporated protoplasts did not indicate any major morphological changes as a result of electroporation. The presence of 4 mM CaCl_2 in the electroporation solution increased protoplast survival and electroporation efficiency (Fromm et al., 1985).

Plant protoplasts from different species require different conditions of electroporation (e.g. field strength or pulse duration) for the introduction of different nucleic acids (Okada et al., 1988). For

example, 65% of rice protoplasts were infected with cucumber mosaic virus (CMV) RNA under stronger and shorter electric pulse (1250 V/cm from 1 μ F condenser). In contrast, electroporation with tobacco mosaic virus (TMV) RNA did not result in infection of rice protoplasts, and tobacco protoplasts were transformed by a weaker and longer electric pulse (750 V/cm, 100 μ F). The kinetics of delivery of DNA by electroporation to monocots was similar to those for dicot protoplasts (Hauptman et al., 1987) except that the expression was generally 10 to 100 times lower in monocot protoplasts (*Triticum monococcum*, *Pennisetum purpureum*, *Panicum maximum*, and *Saccharum officinarum*) than expressed in the dicot species (*Daucus carota*, *Glycine max*, and *Petunia hybrida*).

Three main protocols use electroporation for DNA delivery. One involves the application of short very-high voltage pulses (5 - 200 μ s, 2-10 KV/cm) (Okada et al., 1988). The second approach, employs longer pulses (1 - 50 ms) of lower voltage (200 - 800 V/cm) (Lindsey and Jones, 1989d). The third experimental approach involves a combination of PEG and electroporation (Hauptmann et al., 1987). Carrot, tobacco and maize protoplasts were electroporated (200 - 350 V, 54 ms) in presence of pNOSCAT or pCaMVCAT (Fromm et al., 1985). A short high voltage pulse produced approximately the same CAT activity as a longer pulse at lower voltage. So, the pulse length can be between 30 and 230 ms. Also, Fromm et al. (1986) transformed maize cells by transferring a chimaeric gene encoding neomycin phosphotransferase (CaMVNEO). Tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV) RNAs

have been introduced separately into tobacco mesophyll protoplasts by electroporation (Nishiguchi et al., 1986); optimal infection was achieved with several direct current pulses of 90 μ s/ 5 to 10 KV/cm). Cucumber mosaic virus (CMV) RNA was used to study electroporation conditions suitable to transform rice protoplasts (Okada et al., 1988). Sewart et al. (1987) electroporated carrot protoplasts with a construct containing a heterologous promoter (from the maize zein gene) fused to the bacterial gene for CAT. The GUS gene (pBI 221; see Chapter 2; Fig. 2.1 B) was introduced into *Alnus incana* protoplasts by electroporation (Seguin, 1988).

Stable transformation has been achieved for many plants which can regenerated from protoplasts such as, tobacco (Bates et al., 1988), maize (Carol et al., 1988), tomato (Bellini et al., 1989), and sugarbeet (Lindsey and Jones, 1989d). Tobacco protoplasts were electroporated with a high-voltage, short pulses (200 V/cm, 250 μ s), and transformed plants were obtained (Bates et al., 1988), by using pMON200 which contains the NPT II gene. Also, transformed maize plants regenerated from transformed protoplasts were obtained after a high voltage (200 - 300 V, 500 - 750 V/cm) electrical pulse was applied to the protoplasts (Carol et al., 1988), incubated with two plasmid constructions, pDP23 and pMPI which both carried a NPT-II gene. Southern analysis of DNA extracted from callus and plants indicated the presence of the NPT-II gene. Tomato protoplasts were transformed and transgenic plants were achieved (Bellini et al., 1989) using plasmids pABDI and pGHI which contain kanamycin and chlorsulfuron

resistance genes.

DNA delivery by electroporation and PEG treatment has been reported (Vasil et al., 1988; Planckaer and Walbot et al., 1989). Seguin and Lalonde (1988) reported that when protoplasts were treated with PEG and heat shock, 560 protoplasts were transformed (1.5×10^6) but without the PEG, 88 protoplasts were transformed. A maximum transformation frequency of 25% was obtained from electroporated rice protoplasts incubated with plasmid (CaMVNEO) carrying the NPT II gene (Zhang et al., 1988); this result was significantly higher than those of 20% for tobacco (Nishiguchi et al., 1989), 5% for Maize (Rhodes et al., 1988), 2% for tobacco (Shillito et al., 1985), 1% for maize (Fromm et al., 1986), and 0.2 - 2% for tomato (Bellini et al., 1989).

GUS expression was detected as early as 8 hr after electroporation and appeared to continue for at least 48 hr (Seguin and Lalonde, 1988). CAT activity also was detected in protoplasts as early as 6 hr after electroporation and maximum activity was observed at 24 to 36 hr (Fromm et al., 1985). Two plasmids were used (CaMV 35S-cat-nos and pCaMV 35S-gus-nos) to transform potato protoplasts (*Solanum brevidens* and *Solanum tuberosum*); signals first appeared after few hours and maximum expression occurred about 24 - 48 hr after electroporation (Jones et al., 1989). Sugarbeet (*Beta vulgaris* L) was transformed by electroporation (3 pulses, 500 V/cm, 99.9 μ s) of suspension cultured protoplasts (Lindsey and Jones, 1989d), by using supercoiled pHP23 carrying a NPT II gene. The transformation was confirmed by Southern blot

analysis.

Protoplasts isolated from embryogenic callus cultures derived from immature embryos of *Zea mays* L were transformed by electroporation (450V/cm for 12 ms), by using two plasmids (pALN and pCaMV₁LN), both contained luciferase gene with promoters, 35S RNA and the maize *Adhl* promoters respectively (Planckaert and Walbot, 1989). Electroporation has also been used to stimulate the transformation of protoplasts by *Agrobacterium* (Chand et al., 1989); *Solanum dulcamara* protoplasts were mixed with *A. rhizogenes* then exposed to electric pulses (two pulses of 600 V/cm⁻¹ for 2 ms). Co-cultivation of freshly isolated cell suspension protoplasts of *S. dulcamara* with *A. rhizogenes* for comparable periods failed to transform protoplasts. However, electroporation of protoplasts with *Agrobacterium* followed by a post-electroporation incubation period stimulated transformation. Thus, in conclusion optimal conditions for transformation by electroporation vary enormously and have to be tested on any particular source of protoplasts.

4.2.4 Microinjection

The microinjection technique was developed independently by both A. Graessman and E.G. Diacumakos in 1970 for animal cells and has now become a routine method for the introduction of small molecules, macromolecules (DNA, RNA, protein), organelles and virus particles into a wide range of animal and plant cells (Steinbiss and Stable, 1983; Reich et al., 1986; Griesbach, 1987; Toyoda et al., 1988; Celis, 1984; O'Keefe et al., 1989; Wolff et

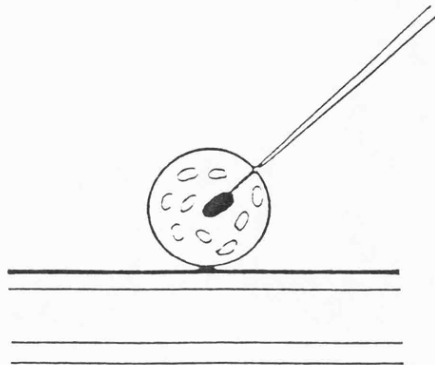
al., 1990). Foreign genes were introduced into different animal cells such as fertilised *Xenopus* eggs (Rusconi and Schaffner, 1981), *Drosophila* embryo (Rubin and Sprading, 1981), *Xenopus* oocytes (Maller et al., 1986; Dash et al., 1987 and Sagata et al., 1988), murine oocytes (O'Keefe et al., 1989), quadricep muscles of mice (Wolff et al., 1990). For example, fertilised eggs of mice were injected with a gene coding for human growth hormone; 70% of the mice that stably incorporated the gene showed a high concentration of human growth hormone in their serum and grew significantly larger than control mice (Palmiter et al., 1983). Also, foreign genes have been introduced and expressed in the pronuclei or nuclei of eggs from superovulated rabbits, sheep and pigs (Hammer et al., 1985). Recently, the quadricep muscles of mice were injected in vivo with either pRSCAT or pRSVCAT or pRSVCAT DNA plasmid. These vectors containing genes for chloramphenicol acetyltransferase, luciferase, and β -galactosidase. The expression was confirmed by CAT activity, cytochemical staining for β -galactosidase, luciferase activity and Southern blot analysis (Wolff et al., 1990).

The success of animal cell microinjection has encouraged researchers to apply these techniques to plant cells (Steinbiss and Stable, 1983; Reich et al., 1986; Griesbach, 1987; Toyoda et al., 1988). One of the difficulties of injecting plant cells has been the inability to easily target specific cellular compartments for injection (Crossway et al., 1986). Reich et al., (1986) used a non-toxic, organelle-specific stain in

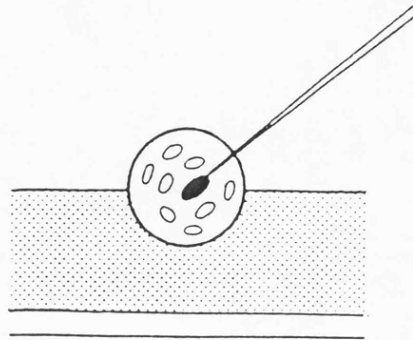
combination with microinjection. Frequencies of 26% have been achieved by the microinjection of plasmid DNA into Hoechst 33258 stained nuclei of alfalfa protoplasts. Lucifer yellow (a very sensitive fluorescent dye) was injected into tobacco protoplasts (Steinbiss and Stable, 1983), about 30% of the injected cells survived the injection procedure and underwent a first division; the protoplasts were held down by poly-L-lysine (Fig. 4.2 A). Evacuation of protoplasts before microinjection to reduce the possibility of inadvertent vacular puncture was reported (Griesbach, 1987). An efficient method, called 'the culture plate method' was devised for microinjection of foreign materials into nuclei of callus cells. This method consists of a dish containing immobilised protoplasts and a simple chamber that maintains sterility and humidity during injection (Fig. 4.2 B; Aly and Owen, 1987; Toyoda et al., 1988). Tobacco protoplasts injected with plasmid (pMON200) or Lucifer yellow dye or buffer solution (pH 7.6) up to 3 days after immobilisation all formed colonies. Of 712 cells injected, 78 colonies (11%) were recovered. Fluorescence was observed within the protoplasts injected with the Lucifer yellow dye (Aly and Owen, 1987). By the same method, kanamycin-resistant tomato callus clones were obtained 1 month after injection from single cells whose nuclei were microinjected with fragments of the pE2KX plasmid containing a NPT-II gene (Toyoda et al., 1988). Delivery of materials into cells had been monitored using many methods, silicone oil was injected into the nuclei of callus cells formed a spherical droplet within nuclei. FITC-albumine also, has

Fig. 4.2 Methods for the microinjection and culture of plant protoplasts, by (A) immobilising protoplasts, or (B) nurse and hanging-drop culture (from Draper et al., 1988).

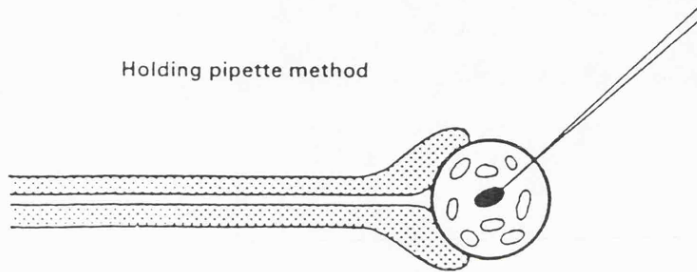
A Poly-L-lysine method



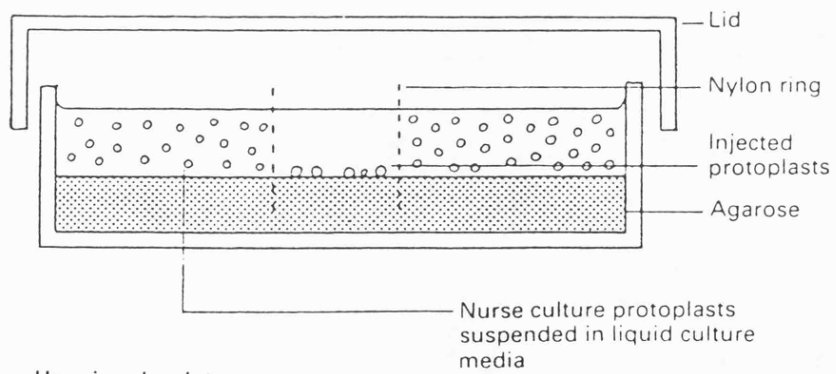
Agarose method



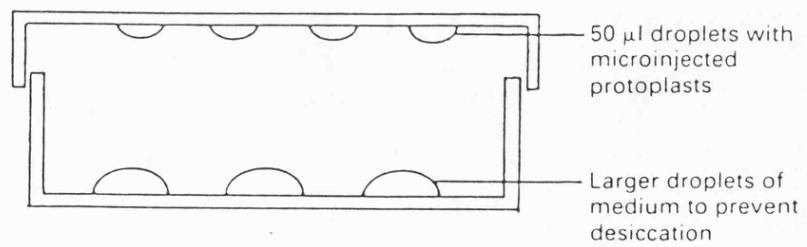
Holding pipette method



B Nylon gauze chamber



Hanging droplets



been injected into cell nuclei; fluorescein being detected by a fluorescence microscope. Efficient transformation of multicellular structures was achieved by injection of DNA into early-staged embryoids derived from mass-cultured microspores of *Brassica napus* (Lichter, 1982) and into microspore-derived embryoids (Neuhaus et al., 1987).

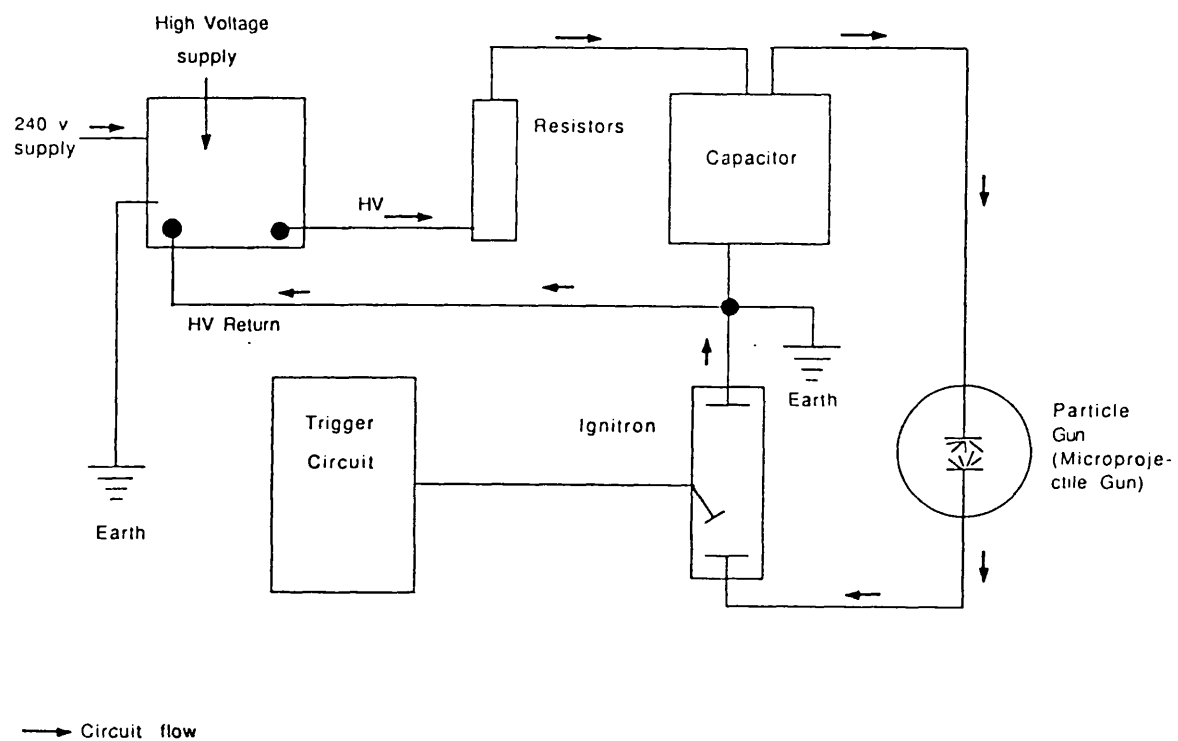
As mentioned earlier, the efficiency of microinjection for transformation is higher in animal cells than in plant cells. For example, following injection into the pronucleus of fertilised one-cell mouse egg the efficiency of transformation was 70% (Rubin and Sprading, 1981) or 25.8% (Wanger et al., 1985). These frequencies compared with 22% (Toyoda et al., 1988) in tomato callus cells, and 14% in tobacco protoplasts. 6% transformation of tobacco protoplasts was achieved by cytoplasmic injection (Crossway et al., 1986), but with the help of the stains to aid intranuclear injection, 26% of tobacco protoplasts was transformed (Reich et al., 1986). One of the problems with microinjection, is that it is technically more demanding than macroinjection (Lindsey and Jones, 1989c). Needle tip diameters should be less than 0.3 μm for intranuclear insertion. By using this size needle, successful insertion rates reached 88 - 92% (Toyoda et al., 1988). The rate of protoplast injection (20 cells per hour; Steinbiss and Stable, 1983) is much lower than in animal cell systems (up to 1000 cells per hour; Graessmann et al., 1980). In another report, up to 7000 animal cells per hour were injected (Yamamoto et al., 1981). With the help of a Vertical-

injectoscope, 200 - 250 protoplasts were injected per hour, and this was higher than using slantwise-injectoscope, which could achieve 100 cells per hour (Toyoda et al., 1988).

4.2.5 Microprojectile-mediated DNA uptake into cells

A gun that fires tungsten microprojectiles loaded with plasmid DNA into cells is one of the most significant developments in the area of gene transfer (Fig. 4.3; Klein et al., 1987). The use of a microprojectile as a DNA carrier might overcome some of the difficulties associated with PEG, calcium phosphate, electroporation and microinjection; these include the limited host range of *Agrobacterium* and the low efficiency or difficulty of regeneration of whole plants from protoplasts. Since the microprojectile gun technique appears to be adaptable to a wide variety of plant tissue, large numbers of cells can be treated simultaneously and it is applicable to intact, regenerable tissues (Christou et al., 1988). The microprojectile gun has enabled researchers to introduce nucleic acids into hitherto non-receptive cereal tissues and cells, and could be applied to intact totipotent tissue and organs of cereal crop species (Karthi et al., 1989). Recipient cells probably have to be in an active state of cell division in order for the introduced gene to be expressed, since mature zygotic as well as somatic embryos failed to reveal any gene expression (Karthi et al., 1989). Meristematic immature soybean seeds were bombarded by plasmid pCMC1100, which contained a GUS gene and pCMC1022, which contained a NPT-II gene (McCabe et

Fig. 4.3 Stylised wiring circuit of the microprojectile gun unit (designed by Mr N. Griffiths, Botany Department, Leicester University).



al., 1988). Approximately 2% of shoots derived from these meristems via organogenesis were transformed. Several factors might affect the efficiency of foreign gene delivery by this technique, such as: physiological state and morphological character of the cells (cell wall thickness, size of cell aggregates, DNA-carrying ability of the tungsten particles, effective penetration into the cells without excessive damage, and the degree of expression of the foreign gene in the bombarded cells (Wang et al., 1988). Transient expression of the CAT gene (pCamVCAT-L) has been detected in cultured barley cells (*Hordeum vulgare* L. cv. Hearland) following the introduction of the gene by microprojectile bombardment. CAT activity was detected as early as 20 hr after bombardment. The expression of CAT activity could be increased with increasing concentration of DNA (Karthä et al., 1989), whereas Wang et al. (1988) reported that an excess of DNA in the DNA-particle mixture decreased the efficiency of the gene transfer.

This technology has its advantages over electroporation which requires large quantities of DNA (Karthä et al., 1989). DNA is delivered to cells in a form that can directly be stably integrated into the *N. tabacum* genome (Klein et al., 1988). It is thought that the DNA is carried into the cell with an initial velocity of about 430 meters per second, absorbs from the surface of the microprojectile and is transferred by some passive or active mechanism to the nucleus. Alternatively, the microprojectile may penetrate the nuclear membrane (Klein et al., 1987, 1988). Foreign genes (pAI₁GusN, pCamVI₁CN)

were transferred directly into the intact cells (cell suspensions derived from callus) of rice (*Oryza sativa*), wheat (*Triticum monococcum*), and soybean (*Glycine max*) by bombardment (Wang et al., 1988). High levels of CAT activity were observed when microprojectiles coated with pCamVI,CN were accelerated and penetrated cell walls and membranes of maize cells in suspension culture (Klein et al., 1988).

The efficiency of transformation after bombardment was usually high, 80% (transient expression) with immature barley embryos (Wang et al., 1988), 76% (stable transformation) of tobacco with the NPT-II gene and 50% with the GUS gene (Tomes et al., 1990), and 2% of meristematic cells in immature soybean seeds (McCabe et al., 1988). Recently, transgenic tobacco plants and progeny carrying the NPT-II gene and GUS gene were recovered (Tomes et al., 1990), following microprojectile bombardment of tobacco leaves. This result was confirmed by GUS histochemical assay and Southern blots. One transformant demonstrated a Mendelian ratio for seed germination on kanamycin-containing medium while the other transformant had a non-Mendelian ratio. Cell autonomous pigmentation was induced in maize tissues that were not normally pigmented (Ludwig et al., 1990); this was achieved by bombardment with plasmid pHI443 containing the Lc gene (a member of the R gene family responsible for tissue-specific anthocyanin pigmentations in the crown of the kernel). The plasmid was bombarded into different tissues of germinating seedlings of maize (Aleurone, coleoptile, root, leorhiza and marginal leaf hair.

Expression of the R gene was located as red spots which were detected 14 - 36 hr after bombardment. Many types of tissues retained pigments for several weeks. Pigmented cells can be quantified in living tissue simply by counting the number of pigmented cells, and the pigments were seen in the epidermal cell layers of the coleoptile, primary root, mesocotyl, scuteller node, and coleorihiza. Pigmented cells were also observed in the surface cell layers of other immature tissues such as pericarp (both inner and outer layers), culm, husk, scutellum, and anther locules. In the same report, plasmid pPH1459 containing a GUS gene and pHI443 were both bombarded into aleurone cells and the expression of both markers (GUS and Lc genes) was seen simultaneously in the tissue; the red cells retained their pigments when treated with the GUS histochemical stain.

4.2.6 DNA uptake by liposome

4.2.6.1 General features of liposomes

Liposomes are microscopic lipid vesicles. They can be prepared by a variety of techniques, all which involve the dispersion of water insoluble phospholipid molecules in an aqueous phase. They have been used to encapsulate various materials, either small materials such as ions, drugs or glucose to macromolecules including enzymes, RNA, DNA and even chromosomes (Poste, et al., 1976; Pagano and Weinstein., 1978; Matthews et al., 1979; Uchimiya et al., 1981a; Fraley et al., 1979; Machy and Leserman 1983; Chicken and Sharon 1983; Freeman et al., 1984; Shew et al., 1985; Kashiwagura et al., 1987; Ballas et al., 1988;

Rosenberg et al., 1988). Most of the published work on liposome-mediated molecule transfer to living cells or tissue deals with animal systems (Papahadjopoulos et al., 1974; Cohen et al., 1976; Ostro et al., 1978; Schwendener et al., 1984; Cudd et al., 1986; Dijkstra et al., 1985; Barratt et al., 1986; Sechoy et al., 1989; Brown and Silviu, 1990). For example, a protein product was isolated which closely resembled a rabbit globin as a result of encapsulating mRNA for rabbit globin by large unilamellar liposomes (phosphatidylserine) which were then introduced into mouse spleen lymphocytes (Dimitriadis, 1978). Liposomes can be introduced into cells by endocytosis, by fusion with the plasma membrane or by injection. Fraley et al., (1981) used the expression of SV 40 into monkey cultured cells as a sensitive biological assay for monitoring the introduction of DNA via liposomes. Injection of encapsulated-DNA into tissue was reported by Cudd et al. (1986). Megamitochondria were isolated one hour after intravenous injection of liposome-encapsulated acridine orange-DNA complex to mice liver. Recently, a drug (cytosine arabinoside) was encapsulated by liposome and was delivered to CV-1 (monkey kidney fibroblast) with markedly greater efficiency than the free drug (Brown and Silviu, 1990).

An early report by Suzki et al. (1977) showed that polystyrene spheres could be taken up by tobacco leaf protoplasts either individually or as aggregates. Both types of uptake were mediated by invagination of the plasmalemma at the site of adsorption of spheres and resulted in the enclosure of spheres in closed vesicles.

Binding and fusion of positively charged liposomes (LUV) loaded with TMV RNA then incubated with tobacco and petunia protoplasts was reported by Ballas et al. (1988). One of the problems which liposomes can help to overcome is the cell surface charge. Mesophyll protoplasts of tobacco, petunia and cowpea were showed to have a negative charged plasmalemma (Nagata and Melchers, 1978), thus the interaction between the protoplasts and the negatively charged DNA is unlikely (Rollo et al., 1981). Positive and negatively charged and neutral vesicles were examined and DNA uptake was achieved. PEG or other endocytosis stimulators were included in the incubation media (Lurquin, 1981; Cassells, 1978; Matthews, et al., 1979; Lurquin and Sheely 1982; Deshayes, et al., 1985). Dijkstra et al. (1985) reported the effect of liposomes carrying different surface charges with cells. The neutral and positively charged liposomes were largely bound by the same-surface binding sites, while negatively charged vesicles attached mainly to other binding sites. For some cells, such as macrophages the degree of liposome-cell interaction can be improved by increasing the positive surface charge (Schwendener et al., 1984). Many reports demonstrated entrapment without degradation by nucleases. Wilson et al. (1977) demonstrated the uptake of poliovirus encapsulated in synthetic large phospholipid vesicles then introduced directly into the cytoplasm of the cell via fusion. Most (>90%) of the vesicle-associated virus appeared to be encapsulated within the interior aqueous compartment of the vesicles rather than adsorped on the surface.

There are three main types of vesicles, based on their size and unilamellar (single lipid layer) or multilamellar structure. In MLV (large multilamellar vesicles) produced by Reeves and Dowben (1969), the lipids are deposited from an organic solvent in a thin film on the wall of a round flask, resulting in vesicles with diameters ranging from 0.05 - 100 μm (Bangham et al., 1965). The MLV can be sonicated resulting in small unilamellar vesicles (SUV) with diameters from 0.02 - 0.05 μm (Papahadjopoulos and Miller, 1967). The MLV and SUV have been used to entrap small molecules, such as a small restriction fragments (875 bp) of plasmid pBR322 DNA (Wong et al., 1980), polycytidylic acid (Magee et al., 1976), invertase (Gregoriadis et al., 1973), concanavalin (Chicken and Sharom, 1983) or atropine methyl bromide (Kashiwagura et al., 1987). The problem with these two types of liposomes is that they showed a relatively low volume of entrapped aqueous space per mole of lipid. In the case of large DNA and chromosomes, it was found that the majority of DNA associated with the liposomes was not trapped in the aqueous core but held externally by electrostatic forces, and at least 60% remained susceptible to DNA degradation (Hoffmann et al., 1978).

REV, (reverse-phase evaporation vesicles) can be formed from a water-in-oil emulsion of phospholipid and buffer in an excess of organic phase, followed by removal of the organic phase under reduced pressure. Vesicles formed by this technique can encapsulate large macromolecular assemblies with high efficiency, high aqueous space to lipid ratio and have a widely variable

chemistry of the lipid compounds (Szoka et al., 1978). The efficiency of encapsulation ranges from 30% - 60% as compared to less than 15% for other methods of liposome preparation (Papahjopoulos et al., 1980). Recently many authors reported the attachment of proteins to the surface of liposomes in attempts to prepare stable liposomes with high binding-affinity and to enhance the efficiency of liposomes as a delivery system (Afzelius et al., 1989; Private et al., 1989; Hutchinson et al., 1989; Sechoy et al., 1989). Immunoliposomes have been prepared by injecting palmitoyl anti-H2K into liposomes suspension (Sullivan and Huang, 1985).

4.2.6.2 Demonstrating molecule transfer into cells via liposomes

There are many methods of locating the encapsulated nucleic acid within the cells, including fluorescent microscopy (Cudd et al., 1986; Lawaczeck et al., 1987), autoradiography (Rollo, 1981; Ahokas, 1987); electron microscopy (Arvinte et al., 1986; Shew and Deamer, 1985; Lawaczeck et al., 1987; Gad et al., 1988). Methods based on using a fluorescent staining of cells following vesicle-mediated transfer are perhaps the most common. Weinstein et al. (1977) reported fluorescence distributed widely when lipid vesicles containing a high concentration of the fluorescent dye 6-CF were incubated with either frog retina cells or human lymphocytes. A "self-quenching" fluorescence is commonly seen with fluorescent dyes; in dilute solution of 6-CF fluorescence is seen to be proportional to the number of dye molecules

present, but as the concentration is raised above 10 mM the yield per molecule drops off rapidly. Uchimiya et al. (1981) reported a maximum uptake of liposome-encapsulated fluorescein diacetate by *Daucus carota* and *Nicotiana glutinosa* protoplasts which lasted for 3 hr. Liposome-mediated transfer of FDA into protoplasts reached a peak after 1 hr incubation. Kanchanapoom and Boss, (1986) examined the effect of various fluorescent compounds on carrot protoplasts. Carboxyfluorescein, scopoletin, fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RITC), rhodamine 123, and rhodamine B ethyl ester were used to monitor their effect on calcium-induced fusion of fusogenic carrot protoplasts. These protoplasts normally fused at a high percentage (50 - 60%) in response to 10 mM calcium, pH 6.0; however, if cells had been labelled with scopoletin, FITC, RITC, fusion was greatly reduced. In contrast, labelling with Carboxyfluorescein, rhodamine B ethyl or ester rhodamine 123 had no detectible effect on calcium- induced fusion.

A number of agents have been added to the media to stimulate the delivery of DNA encapsulated within liposomes into cells. PEG was added widely (Matthews and Cress, 1981; Lurquin and Sheely, 1982; Rouze, et al., 1983; Freemann et al., 1984), but it may be possible that some DNA can be delivered without using PEG (Rosenberg et al., 1988). Polylysine also induces the binding of liposomes (Gad et al., 1985; Gad, 1982), and can cause the rapid aggregation of large unilamellar vesicle (Gad et al., 1985). Polylysine did not inhibit the CAT enzyme activity an in vitro enzyme-substrate reaction (Rosenberg

et al., 1988), also it was found to be less harmful than PEG with watermelon pollen grain germination (Gad et al., 1988). The fusion of acidic liposomes was induced by Mg^{2+} , Ca^{2+} and polymyxin B (Gad et al., 1982). A quaternary ammonium detergent increased the functional transfer of tobacco mosaic virus (TMV) into protoplasts (Ballas et al., 1988). It may have served a dual function; probably to mediate binding of the phosphatidylcholine/cholesterol liposomes to the plant protoplasts and at the same time, promoted a transient increase in the permeability of the protoplast membrane. A serum albumin fragment (m.w. 9000) induced the fusion of the SUV composed of phosphatidylcholine at low pH (Gracia et al., 1984). Calcium plays an important role in protoplast-liposome interaction. Up to 10 mM $CaCl_2$ has been found to stimulate DNA delivery (Rouze et al., 1983; Kanchanapoom and Boss 1986; Gad et al., 1988)

The delivery of encapsulated tobacco mosaic virus (TMV) RNA in a biologically-active form as a convenient assay for monitoring the delivery of nucleic acids using a radioimmune assay was achieved (Fraley et al., 1983; Rouze et al., 1983; Deshayes, et al., 1985; Rosenberg, et al., 1988; Ballas et al., 1988). *E. coli* plasmid, pLGV23neo, carrying a kanamycin resistance gene was encapsulated into liposomes and incubated with tobacco mesophyll protoplasts. The kanamycin resistance gene was transmitted as a single dominant nuclear marker to the progeny (Deshayes et al., 1985). The screenable marker gene chloramphenicol acetyltransferase (CAT) contained in a plasmid vector pUC8CaMVCAT was encapsulated in LUV and

introduced into tobacco protoplasts derived from either cell suspensions or mesophyll tissue (Rosenberg et al., 1988). Expression of the introduced CAT gene could be found for at least seven days. Liposomes (LUV) bearing a positively charged quaternary ammonium detergent were able to mediate functional transfer of tobacco mosaic virus RNA (TMV RNA) into tobacco and petunia protoplasts in the absence of facilitators such as PEG (Ballas et al., 1988). Functional transfer of TMV RNA was revealed by the appearance of specific capsid polypeptides within 48 hr after transfection. Under optimal condition, about 30% of the recipient protoplasts were transfected with TMV RNA; and this result is the optimum so far in comparison with 11% (Uchimiya & Harada, 1981), 12% (Matthews & Cress, 1981), 15% (Lurquin & Sheely, 1982) and 20% (Ohgawara et al., 1983).

Recently, an efficient and reproducible method for RNA transfection, using a synthetic cationic lipid, incorporated into a liposome (lipofecting) was reported (Malone et al., 1989). Transfection of 10 ng to 5 μ g of *Photinus pyralis* luciferase mRNA synthesized in vitro into NIH3T3 mouse cells yields a linear response of luciferase activity. The procedure can be used to efficiently transfect RNA into human, rat, mouse, *Xenopus*, and *Drosophila*.

4.3 DNA UPTAKE INTO PLANTS VIA POLLINATION

In the last 20 years, many methods were used in attempts to introduce foreign genes into pollen nuclei or to transform plants via the pollen tube.

In this section information about direct gene transfer techniques via pollen grains is presented and the advantages and disadvantages of each method are discussed.

4.3.1 Germination of pollen grains in the presence of exogenous DNA

The methods used in attempts to transform plants via pollen grains either used plant genomic DNA, bacteriophage DNA or plasmid DNA.

In early experiments, high molecular weight genomic plant DNA gene containing a specific dominant character was used to transform plants of a different phenotype via pollination. It was hoped that a single gene out of a large number of genes present in the media could be taken up by the pollen grain and cause transformation; this system lacked a selection marker. For example, tumour formation has been used as biological test for the possible transfer of exogenous gene material by pollen. A genetic tumour is formed after a gene in *Nicotiana langsdorffii* was combined with a gene in *N. glauca* (Hess et al., 1975). In a similar approach an anthocyanin gene was transferred between two petunia varieties. However, attributing any red colour to possible gene transfer needs a more vigorous proof (Hess, 1980). Resistance to common rust was transferred between different maize varieties. The experiment was repeated but no resistant seedlings were obtained among thousands of offspring (De Wet et al., 1985). Other genetic characters have also been transferred between distinct maize varieties (Otha, 1986). Although these experiments are interesting there have been

no rigorous molecular proofs that gene transfer had actually occurred.

Procaryotic genetic material was used in several experiments to transform plants via pollination. In this system the number of genes present in the media was limited unlike the previous system. The *E. coli* β -galactosidase gene from Phage $\phi 80$ lac^+ and λlac^+ was transferred to petunia (Hess, 1978, 1979). The growth of petunia seedlings was better on both lactose and galactose selection. A bacterial gene isolated from *E. coli* containing a kanamycin resistance factor was also used to transform petunia. Some plants appeared initially to be transformed but eventually failed to grow in the presence of selective agent. It was possible that the rescued plantlets did not take up any plasmid, but were influenced by their neighbours, or the plasmid was taken up but was lost during vegetative growth (Hess, 1981). As with the experiments using plant genomic DNA these authors provided no strong molecular evidence for gene transfer. In addition, it is very unlikely that procaryotic genes will ever be transcribed in plant cells unless they are close to a very strong plant promoter.

In the early 1980s, engineered foreign genes coding for resistance to kanamycin were developed. Circular and linear forms of pABDI containing a kanamycin resistance driven by a plant promoter were used to try and transform tobacco pollen (Negruitu, 1985). Plasmid pMON273 carrying an NPT-II gene was also used in attempts to transform maize (Booy et al., 1989). These experiments were not successful and as before lacked a full proof of gene

transfer.

4.3.2 Transformation of pre-germinated pollen

Other methods for the transformation of pre-germinated pollen using microinjection of pollen tube nuclei have been attempted (Hepher et al., 1985). The pollen of some important crop species, such as Gramineae, is trinucleate at the time of germination. It was found that pollen germinated in vitro penetrated the stigma with low efficiency because the tube was so long as a result of the time taken for the nuclei to migrate into the tube and become accessible to injection. Injection into the ovary at the time immediately prior to pollen tube entry may be a possible way to achieve transformation. It could be important to only inject DNA at this late stage to reduce unnecessary degradation of the DNA in the ovary. This technique was quick and easy to perform and relatively large amounts of DNA could be injected with no deleterious affect on subsequent seed set (Hepher et al., 1985). Again successful transformation was not reported.

Another method for pollen transformation used liposome-encapsulated plasmid (Ahokas, 1987 and Gad et al., 1988). Ahokas's work is not convincing as the pea pollen and the tubes appeared to be burst in the photographs, there were no molecular proofs and also there have been no reports indicating that the work is repeatable. Watermelon pollen germinated with liposomes (LUV) resulted in the binding of the vesicles to the pollen tube and also fusion to the pollen membrane (Gad et al., 1988). Watermelon pollen tubes did not grow very

well and they had swollen tips. Co-culture of pollen with *Agrobacterium* (C58 or ACh5) was also attempted (Jackson et al., 1980). The seedlings were examined for octopine and nopaline synthesis and gave a negative result. DNA loaded microprojectiles were used to transform maize pollen and it was claimed that 3 transformed plants were recovered (McCabe et al., 1987). This was taken to indicate successful transformation of these plants but to date this work has not been repeated. Tobacco pollen was bombarded with a chimeric gene containing a pollen-specific promoter (52-GUS-NOS3') from tomato (Twell et al., 1989). These pollen grains showed a strong transient expression of the GUS reporter gene. In contrast, only pale blue stained pollen grains were observed following bombardment with pBI221 (containing the CaMV35S-GUS-NOS' gene fusion), which was not different from the background staining of unbombarded pollen. The transient expression was demonstrated by assaying activity up to 48 hr after bombardment (Twell et al., 1989).

4.3.3 Transformation via the 'pollen tube' pathway

Methods for transferring foreign genes *in vivo* using the 'pollen tube' pathway were reported with rice (Luo and Wu, 1988), and wheat (Picard et al., 1988). The general approach here is to transfer foreign genes via either the open tube, as with rice florets, or adding the plasmid after pollination, as with wheat (see Chapter 5; section 5.1.1.3).

4.3.4 Summary of background

There is thus some doubt in the literature as to whether germinating pollen can act as a vehicle for transformation. The absence of strong selectable genes in many experiments and an accurate method of transformant detection for most of the experiments, make it difficult to prove whether the phenotypic changes were due to transformation or due to other reasons such as contamination or presence of other enzymes in the plants. This being the case, much of this section of the thesis set out to devise experiments to re-test some of the previous transformation methods using small plasmids with a very strong selectable marker gene.

4.4 POLLEN AS A TARGET FOR TRANSFORMATION

Pollen offers an ideal target cell for direct gene transfer (Otha, 1986; Ahokas, 1987; Picard et al., 1988; Luo and Wu, 1988). As mentioned earlier in Chapter 1 (section 1.8.2), pollen of some species is amenable to work with as it can be collected in large quantities and can be stored for relatively long periods of time. Also, any system of using pollen as a vehicle for transformation would avoid alteration to the nuclear genome and reduce the chance of genetic chimaery since the transformant will be derived from a single cell. The mature or pre-germinated pollen can be easily placed onto the stigma and fertilisation achieved (see Chapter 3; section 3.9.2.1 and 3.9.3.1). If foreign genes can be carried down to the ovule by a pollen tube, the transformation could be

obtained even if a large number of pollen tubes can not germinate and penetrate or stop growing in the style.

4.4.1 The possibilities and difficulties of DNA uptake by pollen

As mentioned in section 4.1, the major problem in cell/pollen transformation is nuclease activity. The DNA often degrades completely within 5 - 15 min (Matousek and Tupy, 1983; Negrutiu et al., 1986; Westhuizen et al., 1987). The pollen tube emerges from the pollen grain between 15 and 30 min which means that any DNA added at the beginning of the germination period will be degraded before the emergence of the pollen tube. If a high concentration of DNA is incubated with pollen and some taken up by endocytosis (see Section 4.4.2), then the long distance between the tip (probable site of entry) and the generative cell (which is not in a fixed position) may be a problem. If a high concentration of DNA can be mixed with pollen straight after the emergence of the tubes this may guarantee that any internalised DNA will be driven to the generative cell by cytoplasmic streaming; however, the main question is whether the generative cell is capable of incorporation of foreign genes. There are regions in the genome of a non-dividing cell which may be active and thus be possible sites of DNA integration such as nucleolar organising region. Integration may be possible at the time of the 2nd mitotic division resulting in the two sperms; unfortunately the time of this division is not always known. If the generative cell is transcriptionally inactive, then transformation is likely to be impossible.

Getting sufficient DNA into pollen tubes may be a problem and will be addressed in this thesis. A second strategy is possibly to get large amounts of DNA into the cytoplasm and use the pollen tube as a 'delivery chute' down to the ovule. In this case there is no attempt to transform the pollen, only the zygote. DNA uptake may be achieved by many methods that have been extensively used for direct gene transfer, such as incubation with PEG, electroporation, and microprojectiles.

One preliminary objective of the work in this chapter is to investigate the permeability of the pollen tube to DNA or liposomes, and to re-test some of the previous transformation methods using small plasmids with very strong selectable or screenable marker genes such as NPT-II, GUS, or CAT. A second objective will be to investigate the effect of DNA on pollen germination and whether the transformation methods such as incubation with PEG, liposomes or electroporation reduce or destroy the pollen germination and fertilisation system. Moreover, the possibility of detecting nucleic acid in pollen tubes with radiolabelled plasmid, or without the use of radioactivity by enzymatic incorporation of biotin into DNA will be tested. A final objective will be to develop a system to select any transformed seedlings resulting from the transformation experiments.

4.4.2 Demonstrating DNA uptake

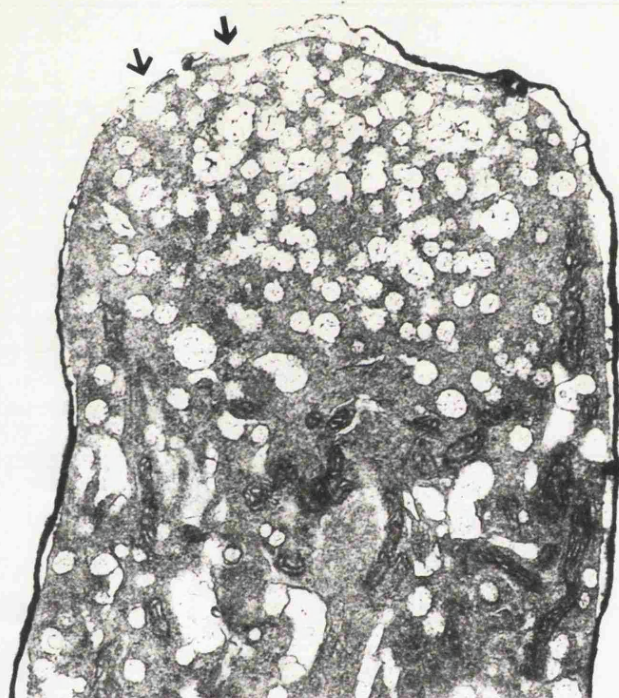
One main objective of this work was to demonstrate, using physical methods that DNA is getting internalised by the pollen tube using different uptake procedures. The

pollen tube grows from the tip which is the only region of the gametophyte with active wall formation. The tip determines the diameter of the tube and influences its direction of growth. The pollen tube tip is bounded by a cell wall. This is usually described as fibrillar. The thickness of the wall at the tip in in vitro-germinated pollen is thin and discontinuous (Fig. 4.4; Steer and Steer, 1989), or the tip may even be devoid of cell wall (Heslop-Harrison, 1980). The pollen tube of *Lilium* possesses only a single layer of wall (Rosen et al., 1964). Thus, because the cell wall at the pollen tip has the characteristic of being discontinuous, or in some species absent, any macromolecule such as DNA or small particles such as liposomes, could be theoretically taken up by endocytosis. In this process the macromolecule adheres to the plasma membrane (absorptive endocytosis) and an invagination forms, this becomes pinched off so taking the macromolecules into the cytoplasm in a membrane-bound vesicle.

4.4.3 Selection techniques for pollen and tissue of pea and tobacco

In parallel with these experiments aiming to optimise DNA uptake into pollen tubes it was also important to develop methods which would allow the selection of transformed pollen or seedlings, following fertilisation.

Fig. 4.4 Longitudinal section through the tip of the
Tradescantia virginiana pollen tube.
Note that some parts the wall (see the arrows)
are discontinuous (from Steer and Steer, 1989).



4.4.3.1 Selection of transformed plants

The best marker gene for the selection of transformed plants was evaluated by transforming pea explants with oncogenic *Agrobacterium* strains harbouring binary vectors.

Conditions for the selection of transformed tobacco plants which have been a comparative system in this project are widely evaluated; genetic transformation and regeneration has been achieved previously by either *Agrobacterium* (Marton et al., 1979; An G., 1985; De Frammond et al., 1986) or by direct DNA uptake into protoplasts via liposomes (Deshayes et al., 1985) or microinjection (Crossway et al., 1985), or electroporation (Nishiguchi et al., 1986).

The natural resistance of germinating seedlings and growing pollen to selective antibiotics was determined in order to decide what level to employ as an effective selection. Since the work was carried there have been some recent reports on a gene expression in pollen cells that are relevant to this thesis.

4.4.3.2 Selection marker genes active in pollen

Transcription and translation occur in the microspore after its formation and during its subsequent development. Mature pollen grains contain mRNAs and protein that were synthesized during development and tube growth utilising the ribosomes and tRNA made and accumulated during pollen maturation. Many types of pollen contain all the proteins, mRNA and ribosomes to

enable rapid germination and initial tube growth on the stigma (Mascarenhas, 1966, 1989; Mascarenhas et al., 1984; Frankis and Mascarenhas, 1980). In *Tradescantia paludosa*, there are two groups of mRNAs with respect to their synthesis (Stinson et al., 1987). The mRNAs of the first group represented by the pollen-specific clones were synthesised after microspore mitosis and increase in concentration up to maturity. This pattern of accumulation would seem to suggest a major function for these mRNAs during pollen germination and early tube growth. The second group of mRNAs, which would include mRNAs like the actin mRNA, accumulate soon after meiosis and reach a maximum by late pollen interphase and decrease thereafter. ADH and β -galactosidase are examples of gene expression in different stage of pollen development (Stinsen and Mascarenhas, 1985 and Singh et al., 1985). The activity of alcohol dehydrogenase (ADH1) was initially detected in maize soon after the tetrads began to break apart. The transcription of the *adh1* gene from the haploid genome occurred very soon after meiosis is completed. ADH activity increases at a constant rate thereafter until microspore mitosis when an increase in the rate takes place which lasts until generative cell division (Stinsen and Mascarenhas, 1985). The synthesis of β -galactosidase during *Brassica campestris* pollen development results from the transcription of the haploid genome. The enzyme activity is first detected in the young microspores by early vacuolate period. A second period of increased enzyme synthesis occurred prior to generative cell division, although the rate is reduced in mature pollen

(Singh, 1985). Steady-state levels of LAT52 mRNA were detectable in immature anthers of tomato containing pollen at the tetrad stage and increase progressively throughout microsporogenesis until anthesis (Twell et al., 1989). In *Oenothera*, the mRNAs for three pollen specific clones, P1, P2 and P3 were detected in immature pollen and reached their maximum level in mature pollen (Brown, 1988 cited in Mascarenhas, 1989). The ZM13 mRNA is distributed throughout the pollen tube cytoplasm (Hanson et al., 1989). The mature pollen grain of *T. paludosa* contains approximately 6×10^6 molecules of poly(A) RNA. Within the first 30 min of germination and pollen tube growth in culture there is an almost 50% decrease in poly(A) content of the tube. This decrease continues at a lower rate during the subsequent 30 min of culture (Mascarenhas and Mermelstein, 1981). It was not known whether the reduction in the level of poly(A) during pollen germination and early tube growth represented a degradation of only the poly(A) tail of the mRNA or whether it reflected the turnover of entire mRNA molecules (Stinsen et al., 1987).

Transcription may be possible from the genomes of the vegetative and generative cell (two sperms) in addition to other organelles of the pollen grain, such as mitochondria. The main question is whether the transcription occurs in all of these cells in the pollen grain or in some of them? If there is no transcription in the generative cell or the two sperm cells, does it mean that they are not susceptible to transformation? So far, there are no reports regarding which cell within the

pollen transcribes and all the reports which have been published measured the total amount of mRNAs/proteins from pollen and large numbers of pollen grains had to be isolated at each of several development stages to obtain sufficient RNA for analysis (Stinsen et al., 1987), this makes it impossible to recognise the exact source of the mRNAs and more research will have to be done regarding isolating each cell type from the pollen grain to find out which cell is transcribing.

Pollen has a very active DNA repair system. It is suggested that the dehydrated state of mature, dehiscent pollen and the protracted exposure of pollen to solar ultraviolet irradiation could lead to considerable damage to pollen DNA. These factors, together with spontaneous base loss and deamination and exposure to naturally occurring positions in the insect pollen load, necessitate a DNA protection and repair capability in pollen to maintain pollen fitness for competition and fertilisation. DNA repair has been demonstrated in pollen from dicots and monocots (Jackson, 1987, 1988; Heberle-Bors et al., 1990).

4.5 RESULTS

Since the completion of the work outlined in this section of the thesis there have been two reports of pea transformation using oncogenic *Agrobacterium* strains.

Different parts of pea plants have been transformed by *Agrobacterium*. Hussy et al. (1989) transformed the meristematic cells in the shoot apex by stabbing with needles loaded with *A. tumefaciens* wild type strains C58

and ACh5. The C58 bacteria induced the formation of tumours whilst the ACh5 inoculations failed to produce tumours. *A. rhizogenes* wild type 9402 gave rise to transformed hairy roots. *A. rhizogenes* carrying the neomycin phosphotransferase gene NPT-II in Bin 19 was also used in these experiments and the presence of the npt-II gene in transformed roots was confirmed by Southern blots. In experiments by Hobb et al. (1989) tumours were obtained on the cut edge of wounded part of the immature leaflets inoculated with 3 wild types *A. tumefaciens* strains A281, C58 and ACh5. A281 appeared to be the most virulent strain, as determined by the size and number of tumours, followed by C58 and ACh5. Calli from material inoculated with C58 had nopaline present, while A281 had agropine and mannopine present, and ACh5 contained octopine.

4.5.1 Assessing virulence of *A. tumefaciens* strains

Preliminary experiments were undertaken to assess the best *Agrobacterium* strains for transforming pea explants. Two oncogenic strains of *A. rhizogenes* (9402 and AG84) and one oncogenic strain of *A. tumefaciens* (A281) were used to assess the virulence of *Agrobacteria* on pea cultivars FS4 and Orb. Tumours were observed on both cultivars with all three strains, but 9402 and A281 produced the most vigorous tumours. For this reason it was decided that the marker plasmid pBI.1 121 (see Chapter 2; Fig 2.1 A), should be inserted into these two strains resulting in two new strains (A281 pBI.1 121 and 9402 pBI.1 121).

The virulence of the three strains of *Agrobacteria*

originally chosen for the study (A281, 9402 and AG84) was tested on four cultivars of pea (Orb, FS4, Countess and Consort). The controls were inoculated without *Agrobacteria* to ensure that any 'tumour' growth was not damage-induced callus growth. The results are presented in Table 4.3

Extensive tumour growth was observed in all cultivars inoculated with A281, 9402, A281 (pBI.121; see Chapter 2; Fig. 2.1 A) and 9402 (pBI.121) (Fig. 4.5 A). Less extensive growth was observed on specimens inoculated with AG84. No callus was observed on the controls (Fig. 4.5 B). Roots were observed growing from some of the tumours induced by A281, 9402, A281 pBI 121 and 9402 pBI 121 (Fig. 4.5 C), although differences were noted between cultivars. No root growth was observed in any cultivars inoculated with AG84. So, *A. tumefaciens* strain A281 and *A. rhizogenes* strain 9402 are recommended to be used for pea transformation.

4.5.2 The effectiveness of the CaMV35S promoter in pea

The ability of the CaMV 35S promoter to drive GUS expression in pea was tested in the two new strains (9402 pBI.121 and A281 pBI.121). Duplicates of the 4 pea cultivars were inoculated with the above *agrobacteria* and the resulting tumours were screened for GUS activity. Control tumours were taken from cultivars inoculated with A281 and 9402 (which both lack the GUS plasmid).

High levels of GUS activity were observed in all tumours induced by *Agrobacterium* containing the marker plasmid, indicating that the CaMV 35S promoter

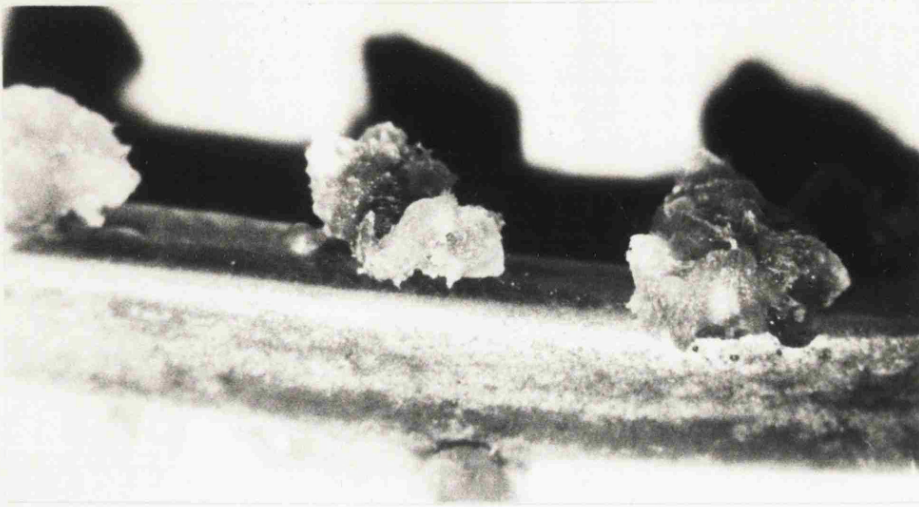
Cultivar	Agrobacterium strain	Tumour growth	Root Growth
FS4	A281	+++	-
	A281 pBI121.1	+++	+
	9402	+++	-
	9402 pBI121.1	+++	-
Orb	A281	+++	-
	A281 pBI121.1	+++	+++
	9402	+++	-
	9402 pBI121.1	+++	+++
Countess	A281	+++	-
	A281 pBI121.1	+++	++
	9402	+++	+
	9402 pBI121.1	++	+++
Consort	A281	+++	+
	A281 pBI121.1	+++	+
	9402	+++	-
	9402 pBI121.1	++	-
FS4	AG84	+	-
Orb	AG84	+	-
Countess	AG84	+	-
Consort	AG84	+	-
Control (stabbed, but not with <i>Agrobacterium</i>)			
FS4	-	-	-
Orb	-	-	-
Countess	-	-	-

Table 4.3 Tumour induction induced by different strains of *Agrobacteria* on stems of four cultivars of pea.

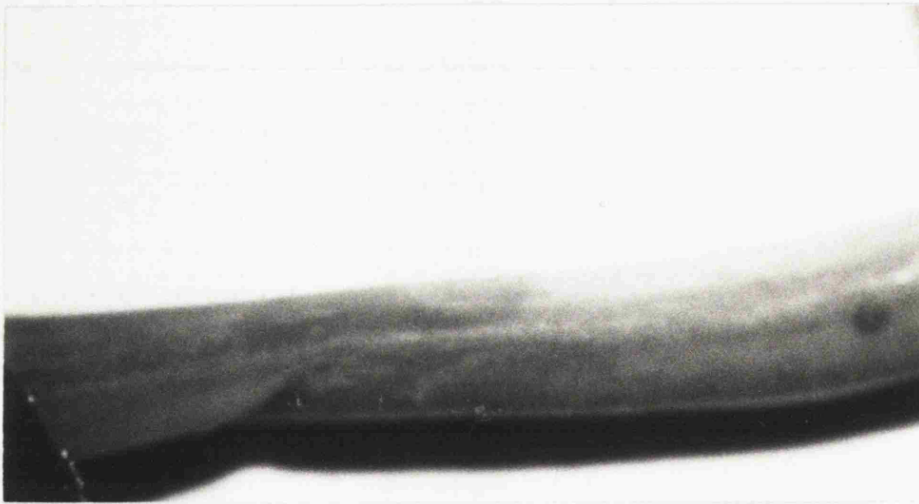
Fig. 4.5 Tumour and hairy roots induction induced by *Agrobacteria*.

- (A) *Agrobacterium*-induced tumour growth on pea stem.
- (B) Control pea stem lacking tumour growth.
- (C) Roots arising from tumours induced by a strain *A. rhizogenes*.

A



B



C



successfully drives GUS expression in pea.

4.5.3 Selection systems

Having identified that the CaMV35S promoter was useful for gene expression in peas, experiments were undertaken to see if resistance to kanamycin could be used as a selection system. The effect of kanamycin on seed germination and pollen development in vitro was determined.

4.5.3.1 Pea seed germination in presence of kanamycin

It was hoped that transformed plants could be selected by the growth of seedlings resulting from fertilisation with DNA-treated pollen on media containing kanamycin.

Control seeds were therefore grown in various concentrations of kanamycin to determine the level at which the plants died. Seeds of Orb and Consort were planted on vermiculite in Kilner jars and 200 ml of autoclaved tap water containing various concentrations of kanamycin (0, 5, 25, 37.5, 50, 62.5, 75 and 150 $\mu\text{g/ml}$). The root length, shoot length, number of nodes and number of lateral roots were recorded (Table 4.4). Seeds of Orb and Consort were also grown on 5 pieces of filter papers and on absorbent towels paper soaked with media containing different concentrations of kanamycin (0, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000 $\mu\text{g/ml}$). The seeds were covered with 2 pieces of filter paper and a further 5 ml of nutrient medium containing kanamycin was added to moisten the

Conc. of km (mg/ml)	Root Length centimeter	Shoot length centimetre	No. of nodes	No. of lateral roots
0	6.4	4.3	4.0	16.5
5	5.3	3.6	2.9	16.1
25	3.9	2.3	2.6	8.6
37.5	7.6	4.0	4.7	11.7
50	6.8	4.4	4.7	15.0
62.5	2.9	2.6	3.4	3.8

Table 4.4 The effect of various concentrations of kanamycin on pea seedling development.

filter papers. Pea seeds were also germinated on agar-solidified MSO media (see Appendix 1) containing the following concentrations of kanamycin (0, 25, 50, 75, 100, 150, 200, 300, 400, 500, 600 $\mu\text{g/ml}$).

The seeds grown on vermiculite in Kilner jars generally bleached at 62.5 $\mu\text{g/ml}$ kanamycin (Fig. 4.6), although a few seeds at higher concentrations remained green. The root length, shoot length, number of nodes and number of lateral roots were longer at some higher concentrations than lower concentrations (Table 4.4), which was unexpected as it was hoped that the higher concentrations should effect the plant more than the lower concentration; also some seeds failed to germinate. Seeds which were grown on filter paper or on rolled tissue paper remained green up to 900 $\mu\text{g/ml}$. Above this level some of the seedlings bleached. At 2 mg/ml, 30% of the seedlings bleached, at 3 mg/ml, over 60% of the seedlings bleached, and at 4 mg/ml, all of the seedlings were bleached. Seeds germinated on MSO solid media (see Appendix 1) did not grow well even in the absence of antibiotics.

In the selection experiments in which the pea seeds were covered partly with water and kanamycin in Kilner jars on vermiculite, the effect of kanamycin on pea seedling development occurred at the growing points of the roots and the shoots. The emergence of these organ systems takes between 24 and 48 hr, and during this time the kanamycin may have lost its efficiency because the kanamycin was not in a sterile environment. The seeds placed on MSO solid media (see Appendix 1) containing

kanamycin gave poor germination possibly because water absorption from the media was very limited.

In summary, the germination of control pea seedlings could not be inhibited in a totally predictable manner. The main problem is possibly the large nutrient reserves in the large cotyledons. Thus, it may be appropriate to explore the use of a screening technique for the detection of transformed plants.

4.5.3.2 Tobacco seeds

Seeds were grown on 10 ml of agar-solidified MSO media (see Appendix 1) containing various concentrations of kanamycin (0, 10, 25, 50, 57, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000 $\mu\text{g/ml}$). 10 transformed seeds [SR1 (CAB GUS)] were germinated as a control under each condition. Between 300 and 500 $\mu\text{g/ml}$, some seedlings bleached whilst some were still green (Fig. 4.7 A). At a concentration of 600 $\mu\text{g/ml}$, all control seedlings bleached whilst the transformed seeds, which were used as a positive control were green and healthy at concentrations between 600 and 900 $\mu\text{g/ml}$ (Fig. 4.7 B, C). So, 600 $\mu\text{g/ml}$ was the optimum concentration to be used for the selection of kanamycin resistance in germinating tobacco seeds.

4.5.3.3 Expression of selection marker genes during pollen development

As mentioned earlier active gene expression during pollen/cell development is probably very important for transformation as it may be required for the integration of incoming DNA. In particular it is very likely

Fig 4.6

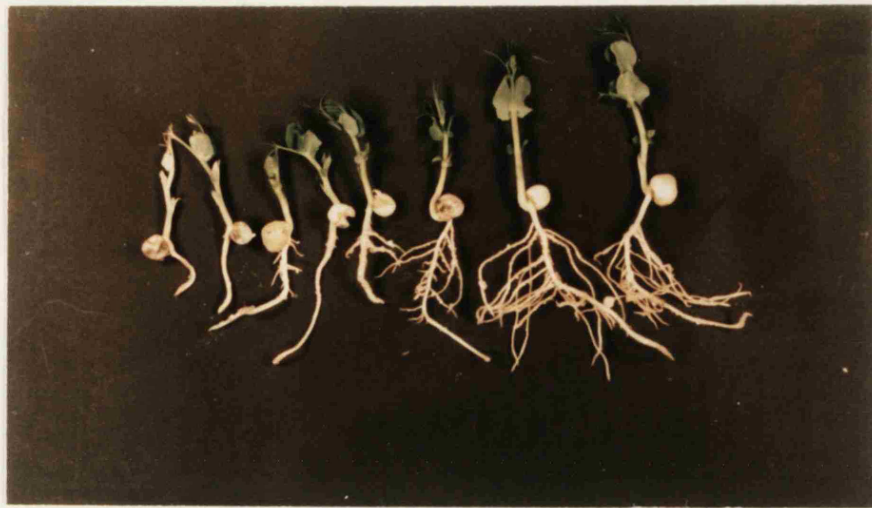
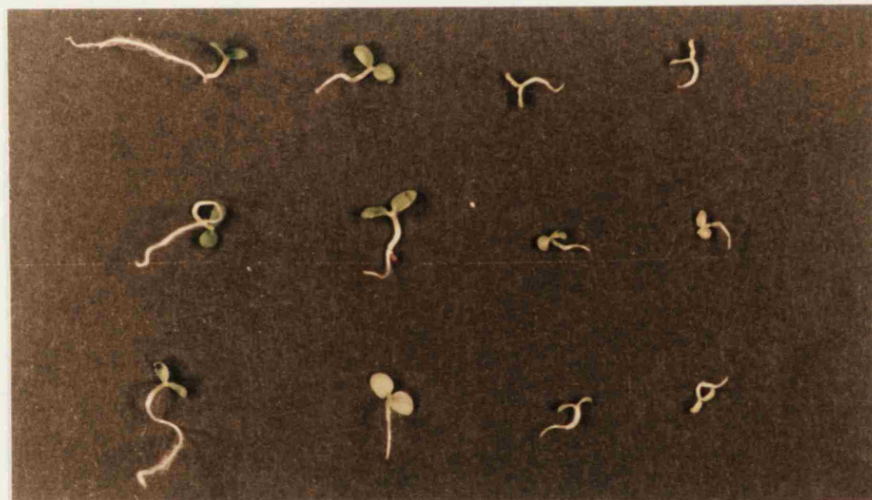


Fig 4.7 A



B



C

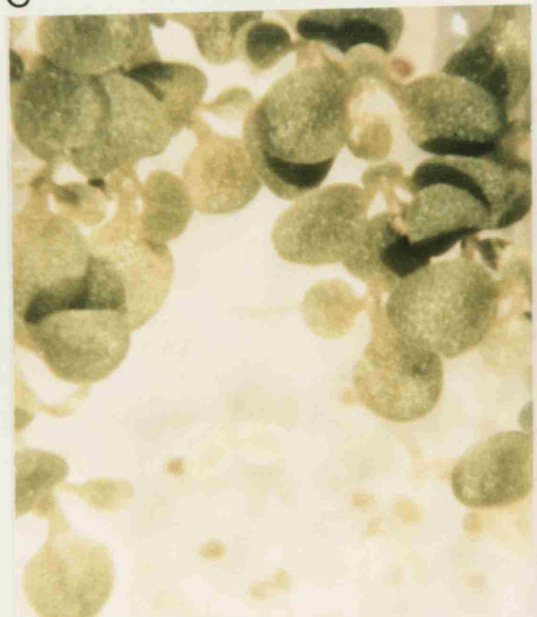


Fig. 4.6 The effect of various concentrations of kanamycin on pea seedlings (kanamycin concentrations from right to left: 0, 5, 25, 37.5, 50, 62.5, 75, 150 $\mu\text{g/ml}$).

Fig. 4.7 The effect of various concentrations of kanamycin.

(A) On tobacco seedlings (kanamycin concentrations, from left to right: 100, 300, 500, 600 $\mu\text{g/l}$).

(B) (C) The effect of kanamycin (600 $\mu\text{g/ml}$) on transformed and untransformed seedlings of tobacco. In (B) the control (untransformed seedlings) is at the left half of the Petri dish. In (C) the control (untransformed seedlings) is at the bottom half of the photograph.

important for competent cell to have DNA synthesis, active transcription or DNA repair. Constitutively expressed foreign genes are normally active in all cells of the transformed plant, but this may not be true of the pollen grain during *in vitro* germination. Kanamycin resistant tobacco plants may transmit the foreign gene but do not express resistance when germinated *in vitro* (Hoffmann et al., 1988). Tomato pollen tube growth was inhibited by the addition of kanamycin to the germination medium (Bino et al., 1987). Pollen from plants containing the NPT-II gene were less sensitive and produced significantly longer tubes at kanamycin concentrations between 200 - 400 mg/l. The effect of pollen tube development was manifest 2 hr after pollen germination possibly because during the early tube growth *in vitro*, transcription is declining. Alternatively the antibiotic reagent may only be gradually absorbed during pollen germination.

4.5.3.4 Tobacco pollen development in the presence of kanamycin

Tobacco pollen from normal plants (untransformed) and transformed plants harbouring a constitutive *npt-II* gene [SR1 (CAB GUS)] were germinated in SM medium at various concentrations of kanamycin (0, 1, 10, 50, 75 or 100 $\mu\text{g/ml}$). Tube growth was recorded between 1 to 6 hr at 1 hr intervals (Table 4.5). There was some inhibition at the concentration of 10 $\mu\text{g/ml}$ for both normal and transformed pollen grains. Few pollen grains germinated at a concentration of 75 $\mu\text{g/ml}$ and no pollen germinated at 100 $\mu\text{g/ml}$. These results indicate that the *npt-II* gene

CONC. OF km mg/ml	TUBE LENGTH OF NORMAL PLANT POLLEN (μ m)						TUBE LENGTH OF TRANSFORMED PLANT POLLEN					
	1hr	2hr	3hr	4hr	5hr	6hr	1hr	2hr	3hr	4hr	5hr	6hr
CONTROL	45	119	222	353	400	606	53	123	175	371	490	724
1	44	95	208	282	356	550	47	104	169	293	542	613
10	0	52	66	120	109	138	0	41	109	149	94	99
50	0	0	0	45	38	57	0	0	0	0	45	74
75	0	0	0	7	19	21	0	0	0	0	9	17
100	0	0	0	0	0	0	0	0	0	0	0	0

Table 4.5 The effect of various concentrations of kanamycin on the tube growth of normal and transformed tobacco pollen.

may be silenced in the pollen of the transgenic plant, and the selection of transformed pollen *in vitro* was not possible.

4.6 DNA UPTAKE TECHNIQUES FOR POLLEN TRANSFORMATION

The vector to be used in the experiments must be small with a very strong selectable/screenable marker gene. High concentrations of plasmid DNA were used in some experiments bearing in mind that some plasmid will be degraded by nucleases. Washing the pollen in germination media is very important to get rid of the nuclease activity which is considered the most difficult problem in cell transformation.

4.6.1 Targets for transformation

There are 3 targets in the pollen tube which may lead to plant transformation:

- i- Integration of the plasmid DNA into a generative nucleus. The generative cell moves from the pollen grain to the tube between 4 and 8 hr either *in vivo* or *in vitro* germination. Plasmid DNA might be introduced into the generative cell to integrate with the genome and as a result the two sperms could be transformed.
- ii- Integration of the plasmid DNA into sperm nucleus. The plasmid might be introduced and integrated during the mitotic division which takes place in the tube and as a result one or both of the sperm cells might be transformed. It would be then hoped that one of them could be taken to the embryo sac and fuse with the egg cell.

iii- Integration of the plasmid into nuclei in the egg and sperm cells at or before the time of gamete fusion in embryo sac. It is possible that DNA present at the time of fertilisation and early embryogenesis would be the most likely to be able to integrate.

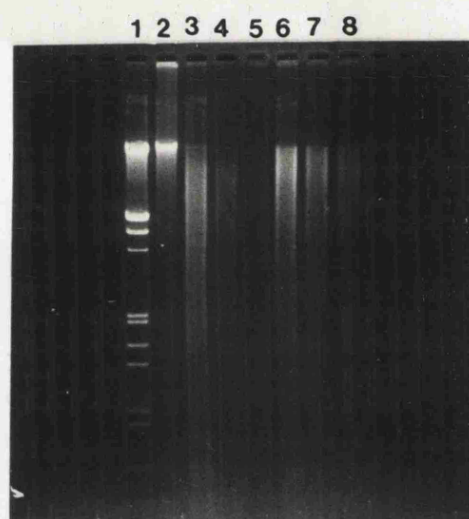
Once a reliable system of *in vitro* germination had been established various methods of obtaining naked DNA uptake were attempted and transformation was assessed by screening for transient expression of a marker gene or selection of transformed seedlings.

4.6.2 Passive uptake of DNA

As mentioned in section 4.1, plasmid DNA does not last for a long time when in contact with pollen in a germination medium before it is degraded. De Wet et al., (1985) germinated maize pollen with maize inbred B73 DNA, the DNA degraded within 15 min after the initial incubation. Negrutiu et al. (1985) germinated pollen in the presence of circular pABDI plasmid (100 μ g/ml), and under standard conditions of germination and pollen growth, the plasmid was completely degraded within 5-10 min of incubation.

In this study, it was found that the plasmid DNA (CaMVNEO ; see Chapter 2; Fig 2.1 C), lasted for 15 min before degradation (Fig. 4.8), taking into consideration that the germination medium was autoclaved and the anther walls were removed and the mixture was incubated at 20°C. Replacing the pollen germination medium before adding DNA to the germinated pollen did not solve the problem.

Fig. 4.8 Effect of washing tobacco pollen on plasmid degradation. Lane 1: lambda DNA digested with *Hind* III and *Eco* RI. Lane 2, 3, 4, 5: incubation of pollen with plasmid after resp. 10, 15, 20, 25 min. Lane 6, 7, 8: DNA after incubation with pollen washed for 15, 20 and 25 min respectively.



4.6.2.1 Tobacco

Pollen from 10 freshly dehisced anthers was placed in 400 μ l of SM (20% sucrose) with 100 μ l of plasmid CaMVNEO (300 μ g/ml; see Chapter 2; Fig. 2.1 C) in a Universal bottle and incubated for 60 min in a growth room at 25°C. The incubation mixture was spun down for 30 sec and then the germinated pollen (95%) was placed on a filter paper drop by drop using a Gilson P 20. In another experiment, pollen was germinated in SM media and every 20 min for 5 hr, the pollen was washed and replaced with fresh media containing 10 μ l pBI.1 121 plasmid (400 μ g/ml; see Chapter 2; Fig. 2.1 A). The germinated pollen was then scraped from the filter paper using a scalpel blade and applied onto emasculated stigmas. 1800 seeds were obtained from such fertilisation experiments. They were sterilised, then plated on MSO media (see Appendix 1) containing 600 μ g/ml kanamycin. After 2 weeks, all the seedlings had bleached and 20 transformed seeds [SR1 (CAB GUS)], used as a control developed as normal.

4.6.2.2 Pea

Pollen from 5 freshly dehisced anthers was placed in 500 μ l of BM and 50 μ l of plasmid pBI.121 (265 μ g/ml; see Chapter 3; Fig. 2.1 A). 110 stigmas of emasculated flowers were pollinated. 21 seeds were obtained and they were screened for GUS activity by histochemical assay. No blue precipitate was seen.

4.6.3 PEG treatment of pollen

Uptake experiments were carried out utilising PEG,

which has the ability to stimulate DNA uptake into protoplasts (see Section 4.2.1)).

In this experiment, the PEG was incubated with germinated pollen in order to disrupt the stability of plasmamembrane structure at the tip which has a discontinuous wall thus probably allowing contact between the plasmid DNA and plasmamembrane.

1153 tobacco seeds were obtained and sterilised and then they were selected (as mentioned in section 4.6.2) and after a week, all seedlings were bleached. The high concentration of PEG (20% and 25%) caused some inhibition of pollen germination (Table 4.6).

These experiments were also repeated with pea pollen in which stigmas were pollinated with pollen treated with 10%, 15% and 25% PEG, but no seeds were obtained. In pea, the fertilisation was affected as the pea pollen is so delicate and the pollen germination was sharply reduced (Table 4.7). The viscosity of this chemical may disrupt the fertilisation process.

4.6.4 Electroporation of pollen tubes

Electroporation creates pores in the plasma membrane. These pores do not close immediately but close gradually facilitating entry of molecules into the cytoplasm (see Section 4.2.3). In this experiment, the electric pulses might create pores on the pollen tube tip which could allow the plasmid to flow into the tubes.

Germinated pollen was electroporated in the presence of plasmid pBI.1 221 gene (see Chapter 2; 2.1 B) which contains the GUS (β -glucuronidase) gene and subsequently

Con. of PEG %	Percent germination
0	89
10	69
15	46
25	39

Table 4.6 Effect of various concentrations of PEG on tobacco pollen growth.

Con. of PEG %	Percent germination
0	79
10	61
15	32
25	26

Table 4.7 Effect of various concentrations of PEG on pea pollen growth.

assayed for transient expression. The substrate used for the histochemical location of β -glucuronidase activity in tissue and cells, was 5-bromo -4-chloro-3-indolyl glucuronide (X-GLUC). This substrate gives a blue precipitate at the site of enzyme activity. The product of glucuronidase action on X-GLUC is not coloured. The indoxyl derivative produced must undergo on oxidative dimerisation to form the insoluble and highly coloured indigo dye. The dimerisation is stimulated by atmospheric oxygen, and can be enhanced by using an oxidation catalyst such as K^+ ferricyanide/ferrocyanide mixture (Lojda, 1970)

Tobacco pollen was placed in 2 ml of SM and incubated for an hour to allow germination. 0.5 ml of the germinated pollen solution was then aliquoted and 20 μ l of plasmid solution (250 μ g/ml) was added. This procedure was repeated three times to produce 4 identical suspensions containing GUS plasmid and germinated pollen. Three of the pollen/plasmid mixtures were then electroporated (pulse duration of 10 μ : 50v, 100v and 150v; one at each setting, Kruss AT 750 electroporator). The unelectroporated sample was designated the control. Pollen was assayed using the histochemical assay (see Chapter 2; section 2.10), but no blue precipitate was visible.

4.6.5 Coating pollen with plasmids

Experiments were performed which attempted to coat pollen with a dry plasmid coat. It was thought that the plasmid might be taken up during the pollen hydration which occurs through the pores and slits in the pollen

grain wall which accommodate the water passage. The water transfers usually from the stigma in vivo or the media in vitro. In this experiment from the plasmid solution which was mixed with ethanol, ether, acetone, chloroform, or Q water.

4.6.5.1 Fertilisation by treated pollen

The aim of this experiment to find out whether the fertilisation was disrupted if pollen was treated with ethanol, ether, acetone, chloroform, or Q water. Tobacco pollen was applied to a glass slide. A drop of ethanol (or other solvent) was dropped onto the pollen and allowed to evaporate. Pollen from each treatment was applied to 3 emasculated stigmas and allowed to set seeds. Between 500 to 600 seeds were obtained from each treatment proving that these solvents did not disrupt pollen structure or the fertilisation process.

4.6.5.2 Transformation by plasmid coated pollen

In transformation experiments, a drop of solvent: plasmid mix was placed onto the pollen and allowed to evaporate (10 - 15 sec). 500 seeds were obtained from the flowers treated with a plasmid:ethanol mix; 650 seeds from plasmid:acetone mix; 611 seeds from chloroform:plasmid mix; 420 seeds from plasmid:ether mix and 953 seeds from plasmid:Q water mix. These seeds were sterilised and plated on selection media containing kanamycin. All seeds bleached on germination.

4.7 MICROPROJECTILE-MEDIATED TRANSFORMATION OF TOBACCO AND PEA POLLEN

4.7.1 Background

The particle gun has been used to deliver DNA into the plant cells (Klein et al., 1987; Christou et al., 1988; McCabe et al., 1988; Wang et al., 1988; Kartha et al., 1989; Tomes et al., 1990; Ludwig et al., 1990), and pollen (Twell et al., 1989).

The desired DNA can be loaded onto gold particles and these can be fired into plant tissue at high speed. Each particle is made of an inert metal and are very heavy. Being inert ensures that they do not react adversely with the plant cell, and being heavy gives them more power. The gold is much smaller than the plant cell so it can enter the cell and deliver its loaded DNA without damaging it, resulting in either transient expression or stable transformation. As mentioned in section 4.2.5, the use of microprojectiles might overcome some of the difficulties associated with other methods of transformation; these include the limited host range of *Agrobacterium* and the low efficiency or difficulty with regeneration of whole plants from protoplasts.

There are many difficulties with pollen transformation, such as nuclease activity, the long time between pollination and fertilisation, difficulties of obtaining fertilisation from pregerminated pollen in some species, and sensitivity of pollen of some species to chemicals such as PEG. These problems make pollen a difficult target for transformation in spite of its

importance as a reproductive tissue. So, it was hoped that the microprojectile could deliver the desired DNA as this system can avoid some of the mentioned problems. These experimental studies were started at the very end of the experimental period. This part of the project was undertaken in collaboration with Mr N. Griffiths, Botany Department, Leicester University.

4.7.2 Results

In this experiment, germinating tobacco and pea pollen was bombarded with pBI 221 (CaMV35S-GUS-NOS-ter; see Chapter 2; Fig. 2.1 B), after various times of incubation (see Chapter 2; section 2.19.5). Immature pollen at the tetrad stage which includes four meiotic products from a pollen mother cell were also bombarded. No blue precipitate was observed either on the pollen grains or pollen tubes or in the pollen at the tetrad stage, after 16 - 48 hr.

4.7.3 Discussion

Most of the genes expressed during pollen development appear also to be expressed by the sporophyte. Recent interest in the male gametophyte resulted in the isolation of pollen-specific-expressed sequences including pollen specific cDNA and genes from several plants. These pollen-specific genes are now being characterised with respect to promoter elements required for male gametophyte-specific gene regulation (Mascarenhas, 1989; Twell et al., 1989). Unfortunately there are not many pollen-specific promoters available but one might expect rapid

progress in identifying those genes/promoters and their functions that are critical in initiating the unique development events during pollen development and maturation (Mascarenhas, 1989). The bombardment of tobacco pollen by pollen specific-promoter LAT5 was used to achieve the first transient expression of gene in pollen and no transformation was achieved when the pollen bombarded with pBI 221 (CaMV35S-GUS-NOS-ter; see Chapter 2; Fig. 2.1 B) (Twell et al., 1989).

In *Petunia hybrida*, P_{A1} , P_{A2} , and P_B promoters were characterised and isolated (van Tunen et al., 1987, 1988, 1989, 1990), CHI enzyme (chalcone flavanone isomerase) is encoded by two genes, *chiA* and *chiB*. *chiA* is regulated by two distinct promoters that act in tandem and are differentially used; the downstream P_{A1} promoter is active in corolla, tube, and mature anther tissue giving rise to a 1.0-kb mRNA, whereas the upstream P_{A2} promoter (440 bp) is active in mature anthers and, more precisely, in pollen grains (giving rise to a 1.5-kb transcript). The *chiB* gene is regulated by a single promoter, P_B (1.75 kb), which is active in immature anther tissue resulting in the accumulation of a 1.0-kb transcript. Recently, *zE19* gene was isolated (Quattrocchio et al., 1990 in press), it contains two distinct promoters which are independently active in endosperm and anthers of transgenic petunia plants.

In addition to the LAT52 gene which was described earlier, there are 4 similar genes which have been isolated from tomato (*Lycopersicon esculentum* Mill.), LAT51, LAT56, LAT58, and LAT59 (McCormick et al., 1987).

Transcripts of LAT56 and LAT59 were present in microspores, pollen grain, pollen tube and in the anther wall. LAT56 encodes a putative 365 amino acid protein (p56) of 40,561 Da with a predicted pI of 8.28. LAT 59 encodes a putative 449 amino acid protein (p59) of 50,893 Da with a predicted pI of 8.36. It was suggested that LAT56 and LAT59 code for pectate lyases which relate to a requirement for pectin degradation during pollen tube growth (Wing et al., 1990). LAT51 and LAT58 were localised in pollen, mature anther and less in green petal (Ursin et al., 1989). The promoters of these genes have not yet determined except for LAT52 (Twell et al., 1989). So, pollen-specific promoters might open up the door for transforming the reproductive organs of the plant (pollen grains and ovules).

4.8 REASON FOR LACK OF TRANSFORMATION OF POLLEN

The integration of the plasmid within the vegetative cell nucleus or the second sperm cell is of little practical value as they are lost in the fertilisation process or during seed maturation. The third target which is the presence of the plasmid at the time of fusion between the egg cell and the sperm is the most likely for transformation especially that it has been proved that the DNA can be introduced into the embryo sac (Picard et al., 1988).

Direct DNA uptake into pollen tubes was not successful in terms of yielding transformed plants. The amount and concentration of DNA used in these experiments, 50 μ l (300 μ g/ml) for passive uptake, 20 μ l (250 μ g/ml)

for electroporation and 25 μ l (250 μ g/ml) for PEG, have previously been found sufficient to transform tobacco protoplasts. With pollen perhaps there are problems with DNA uptake or the time required for growth down the style or with nuclease activity. The time between the pollen incubation, PEG or electroporation and the fertilisation was between 25 - 30 hr and perhaps this time is too long for the plasmid to survive the nuclease activity which released during the pollen germination (see Section 4.1). Following electroporation of pollen no blue precipitate was seen in the GUS assay. This suggested that either no transient expression of the GUS construct occurred during pollen germination *in vitro* or that plasmid failed to enter the tube. Bearing in mind this data experiments were set up to prove that DNA was indeed entering the germinated pollen tube.

4.9 DETECTION METHODS TO DETERMINE DNA UPTAKE INTO POLLEN

4.9.1 Biotin-labelling of DNA to demonstrate its uptake and position in germinated pea pollen.

Detection of nucleic acids without the use of radioactivity is now possible by enzymatic incorporation of biotin into DNA and the subsequent colourmetric detection of biotin (Forster et al., 1985).

Biotin is an essential water-soluble vitamin which is required for normal cellular function, growth, and development. Biotin acts as a co-enzyme in many metabolic reactions including fatty acid biosynthesis, gluconeogenesis and amino acid production. Human and

higher mammals cannot synthesize biotin, therefore, they must obtain the vitamin by intestinal absorption (Said and Redha, 1988).

Biotin or [^3H] Biotin binding uptake by different cells and organisms has been achieved. For example, by *E. coli* K12 (Parkash and Eisenberg 1974; Cicmanes and Lichstein, 1978; Piffeteau et al., 1982; Piffeteau and Gaudry, 1985), isolated rat intestinal cells (Gore et al., 1986), and rat intestinal brush-border membrane (Said and Redha, 1988)

Biotin is photoactivable (Fig. 4.9) and binds to nucleic acid when illuminated with strong light, it will form a stable linkage with single- and double stranded nucleic acids with one biotin couple per 100 to 200 residues (Forster et al., 1985). Biotin can be detected by binding with streptavidin (from *Streptomyces avidinii*) conjugated with an enzyme such as alkaline phosphatase. This is then visualised by incubation with a substrate for the enzyme which generates a coloured product.

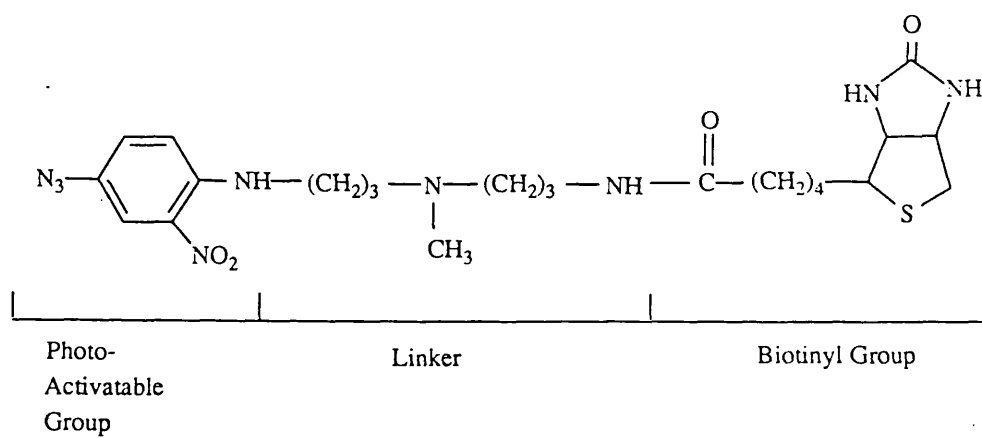
4.9.1.1 Labelling the plasmid

The biotin was labelled to plasmid CaMVNEO (see Chapter 2; Fig. 2.1 C) by placing the mixture under strong light. The labelled plasmid was then extracted, then the supernatant was washed and the plasmid dried, then dissolved in Q water (see Chapter 2; section 2.17.1)

4.9.1.2 Pollen germination in the presence of biotin-labelled plasmid

Freshly dehisced anthers were ruptured with fine

Fig. 4.9 Structure of photobiotin



forceps to release their pollen into a Universal bottle containing 2.5 ml of BM with and without 10% PEG 6000. This stock was distributed to 5 Eppendorf tubes. In the first treatment, the pollen was allowed to germinate for 40 min in the presence of 16 μ l of labelled plasmids, then washed 3 times. DNase (2 μ l DNase/0.5 μ g of DNA) was added and left for 20 min at 37°C to destroy any plasmid which had not been removed by the washes; the tube was flicked every 5 min to mix them. If germination proceeded for more than 40 min it was difficult to burst the tubes. In the second treatment, the pollen was allowed to germinate for 30 min in the presence of 16 μ l of labelled plasmids, then washed 3 times and allowed to germinate for a further hour to show the new growth. In the third treatment, pollen was allowed to germinate in the presence of 16 μ l labelled plasmids for 1.5 hr but no washes were performed. The fourth treatment involved the pollen germinating in the presence of unlabelled plasmids for 1.5 hr. In the fifth treatment, the pollen was allowed to germinate in BM for 1.5 hr.

The pollen was germinated on a shaker at 25°C and observed regularly to check that germination was proceeding normally. The washing was done by spinning the tube for 30 sec removing the supernatant and replacing with fresh media, then the pollen tubes were fixed (see Chapter 2; section 2.8). The labelled plasmid was visualised by adding SAAP (Streptavidine alkaline phosphotransferase) to each slide over the pollen to generate a coloured product (see Chapter 2; section

2.17.2), then examined under the microscope

4.9.2 Results

Five treatments were performed. The first treatment was performed by incubation of the pollen with labelled plasmids, it was then washed and finally treated with DNase. The burst tubes showed labelled plasmid as a red dots inside the cytoplasm; labelled plasmid was not seen outside the tube (Fig. 4.10 A). In the second treatment, the pollen was incubated with labelled plasmids, and then washed. The labelled plasmid stuck to the pollen tube (Fig. 4.10 B) and no background was seen; as the pollen tube grows from the tip, it was interesting to see that no labelled plasmid was visible in the new growth (Fig. 4.10 C). In the third treatment, pollen was incubated with labelled plasmid. The labelled plasmid was seen to be stuck to the pollen grains and pollen tube wall and in the cytoplasm where the tube had burst. In this treatment it was noted also that much background staining occurred (Fig. 4.10 D). The fourth treatment involved incubating the pollen with unlabelled plasmid DNA. In this case the red colour was not associated with either background or associated with the pollen grains or the pollen tubes (Fig. 4.10 E). In the final treatment, pollen was germinated normally. No red colour was visible either on the pollen grain or the pollen tube wall and also no background was seen (Fig. 4.10 F).

The results showed that there was interaction between biotin-labelled plasmid in germination media and

Fig. 4.10 Detection of biotin-labelled DNA.

- (A) Burst pea pollen tube.
Note the labelled plasmids appears as red dots inside the cytoplasm after 40 min incubation (see the arrow; x 950).
- (B) Intact pea pollen tube washed after DNA uptake.
Note the labelled plasmids stuck to the whole tube after 1.5 hr incubation (x 950).
- (C) Washed part way through the pea pollen tube germination procedure.
Note that no plasmid was observed in the new growth after 1.5 hr incubation (see the area between the two arrows; x 950).
- (D) Unwashed pea pollen tubes after DNA uptake.
Note the labelled plasmids stuck, to the pollen grains and pollen tubes and in the cytoplasm after 1.5 hr incubation, also much background staining (x 80).
- (E) Pea pollen tube incubated with unlabelled plasmids after 1.5 hr incubation then burst, to indicate that neither the unlabelled plasmids nor the dye caused the red dots (x 380).
- (F) Pea pollen was germinated for 1.5 hr then burst as a control (x 380).

A



B



C



D



E



F



the germinating pollen. DNase removed plasmid from outside the tube and the media but did not enter the tubes and thus the only DNA signals remaining were likely to be internal. The labelled plasmid (red dots) was observed in the cytoplasm of the burst tubes indicated that the labelled plasmid may have entered the tube before the DNase treatment. No labelled plasmid was seen outside the pollen since it had been removed by the DNase treatment.

Background staining was not seen in the pollen which had been incubated with labelled plasmid and washed; however plasmid stuck to the tube wall had not been removed. As expected no labelled plasmid was seen in the new growth.

In pollen which was incubated with labelled plasmid and not washed, the pollen grains and the pollen tubes appeared red and there was much background staining which showed that pollen adsorped DNA readily. Control pollen or pollen which was germinated with unlabelled plasmid, showed no red colour indicating clearly that the colour was not due to the dye or to the plasmid alone. These experiments suggested that naked plasmid might be taken up by the pollen tubes and more work should be done to know how long the plasmid DNA could survive, and also to protect the plasmid DNA from nuclease attack by using liposomes.

4.10 RADIOLABELLING PLASMID TO DEMONSTRATE DNA UPTAKE INTO TOBACCO POLLEN TUBES

Most early experiments involving DNA uptake into protoplasts utilised radiolabelled DNA including *in vivo*

labelled double-stranded genomic DNA (Uchimiya and Murshige, 1977; Rollo et al., 1980), single-stranded phage DNA (Suzuki and Takebe, 1976) and plasmid DNA (Lurquin and Kado, 1977; Owens, 1979; Lurquin, 1981), and plasmid DNA labelled in vitro using the nick translation technique (Lurquin and Sheehy, 1982; Lurquin and Rollo, 1983). In an autoradiographic analysis of thin-sectioned material, Lurquin and Rollo (1983) showed that plasmid DNA was transferred to the interior of protoplasts. Similar experimental protocols have been employed for the uptake into the protoplasts of fluorescence-labelled DNA (Lurquin, 1979; Lurquin and Rollo, 1983). Although it was possible to determine the percentage of the protoplast population that received donor DNA, using both fluorescence and autoradiography (Rollo et al., 1980; Lurquin and Rollo, 1983), it was not possible to quantify the amount of DNA delivered.

4.10.1 Fractioning the plasmid and radiolabelling

Plasmid DNA CaMVNEO (see Chapter 2; Fig. 2.1 C) was cut by restriction enzymes, then end-labelled with radioactive ^{35}S (see Chapter 2; section 2.18.1). Labelled plasmid was added to 3 tubes (10 μl each) containing 0.5 ml of SM and pollen: In the first treatment, the pollen was allowed to germinate for 30 min, then washed 3 times. DNase was then added and the pollen allowed to germinate for a further 1 hr. In the second treatment, the pollen was allowed to germinate for 30 min, then washed 3 times and allowed to germinate for a further 1 hr. In the third treatment, the pollen was allowed to germinate for 1.5 hr

(no washes were done). In the fourth treatment, the pollen was allowed to germinate for 1.5 hr with no labelled plasmid (control).

The germinated pollen in the above treatments was fixed (see Chapter 2; section 2.8) stuck to slides and autoradiographed.

4.10.2 Results

The first replicate (4 slides) were developed after 2 weeks. The pollen tubes were clearly visible, but the slides were not developed sufficiently. The second replicate was developed after 4 months. The pollen treated with DNase, washing only, or no washes, showed black dots (radioactively labelled plasmid) on the outside or inside the tubes (Fig. 4.11 A, B, C). The pollen which was not treated with labelled plasmid showed clear tubes.

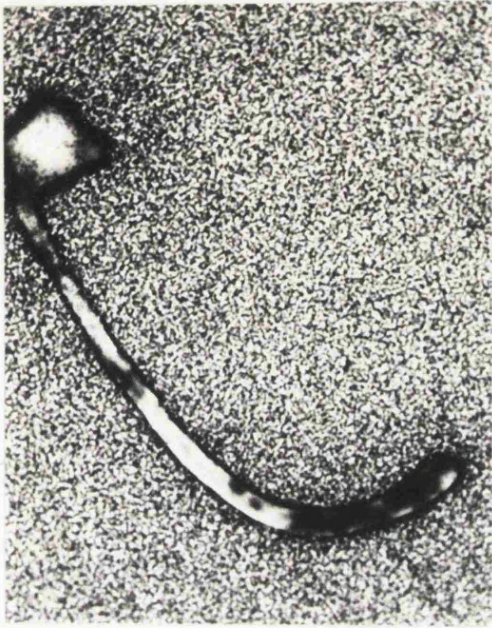
The pollen germinated with radiolabelled plasmids showed a lot of background in the slides; this prevented clear results from being obtained. However, it was noticed that radiolabelled plasmid was not present in some parts of the pollen tube which were thought to be regions containing tube vacuoles.

This work should be repeated and further research should be done such as using of the radiolabelled plasmid to follow the progress of DNA introduced to tissue by microinjection or microprojectiles or pollination with radiolabelled pollen to find out if labelled DNA reaches the egg cell.

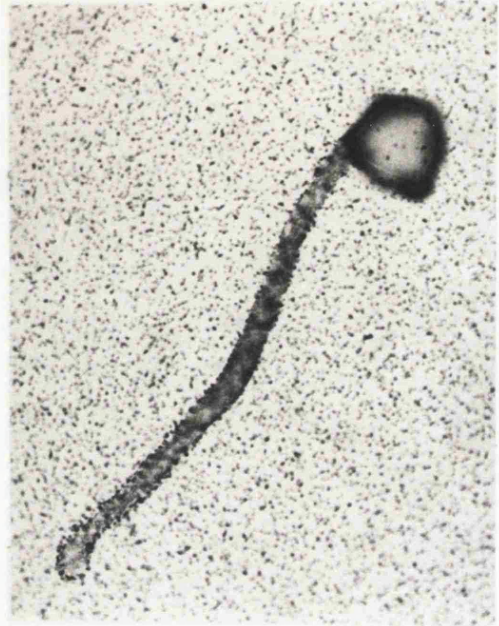
Fig. 4.11 Detection of DNA interacting with pollen using autoradiography.

- (A) Tobacco pollen tube incubated with radiolabelled plasmids after 2 hr incubation then treated with DNase. Note that some of the tube areas are clear (x 450).
- (B) Tobacco pollen tube incubated with radiolabelled plasmids then washed 3 times with media. Note that the radiolabelled DNA is found all over the whole tube after 2 hr incubation. Much of the background is on the slide (x 450).
- (C) Tobacco pollen tube incubated with radiolabelled plasmids for 2 hr and no washes was done (x 450).

A



B



C



4.11 FUSION OF THE TOBACCO POLLEN TUBES WITH LIPOSOMES

Liposomes are a valuable carrier for delivering of nucleic acid into cells. The final aim of these experiments is to investigate the possible toxic effect of the liposome on pollen germination and possible effects on the fertilisation process. The ability of liposomes to protect plasmid DNA from degradation may allow a longer time for DNA to survive inside the pollen tube and could promote integration. For obtaining a pollen fusion with liposomes, the best stage should be determined in pollen tube growth to add the liposomes. As the boric acid is important to pollen growth (see Chapter 1; section 1.7.4), the effect of boric acid should be investigated to make sure that the concentration of boric acid does not affect liposome stability. PEG was used widely in cell transformation as a stimulator of liposome fusion/uptake; in this experiment the precise concentration of PEG to stimulate liposome binding and uptake was determined which at the same time did not harm pollen growth. Finally, the surface charge of pollen tube and pollen grain was investigated in order to treat the pollen tubes with a suitably charged vesicle.

The liposome preparation was very complicated and substantial time was consumed in constructing the different parts of the liposome producing machine, and obtaining the right size of liposomes. This part of the project was undertaken in collaboration with Miss Celia James.

4.11.1 Pollen germination media

The appropriate medium is very important for maintaining the integrity of both pollen and liposome. The chemicals in the pollen media must not affect liposome formation and the osmotic balance must not burst the liposomes. Also the liposome buffer should not inhibit pollen germination.

Pollen germinated in SM medium as a control, gave very good germination. Pollen germinated in liposome buffer gave poor germination; this was due to the presence of EDTA which whilst improving the formation of uniform liposomes strongly inhibited the pollen germination. This buffer also lacked sugar and boric acid. Pollen germinated in 50% SM and 50% liposome buffer gave better germination than in just liposome buffer (Table 4.8; 3 replicates). Eventually a comparison was found which gave good germination and normal liposomes. This consisted of adding 150 μ l liposome mixture to 1 ml of pollen growth media.

4.11.2 Determination of best stage in pollen tube growth to add liposomes

Addition of liposomes to pollen at the right stage is very important as the pollen tube grows when incubated in germination media unlike protoplasts which do not have this characteristic. So, the liposomes should be added at an appropriate time to ensure binding to the pollen tube surface and entering the tube via the tip. Gad et al. (1988) added liposomes to water melon pollen between 60 and 90 min after germination when the generative nucleus

Germination media	Tube length (μm) average of 25 pollen tubes
Liposome buffer	43
50% SM and 50% liposome buffer	150
150 μl liposome buffer in 1 ml SM	420

Table 4.8 The effect of different media on pollen germinated
in the presence of liposomes.

was in the tube. The water melon pollen tube grew slowly unlike pea or tobacco pollen in which the tube grew quickly and the generative nucleus appears in some tubes (25%) in the first 30 min. Ahokas added the liposome suspension after about 10 min from germination.

Liposomes loaded with DAPI-labelled DNA were used to monitor interaction of these vesicles with germinated pollen. It was found that the best time to add liposomes was either at the pollen hydration (during the first 20 sec), or during the 5 min after the pollen hydration. The liposomes appeared to stick both outside and inside the tubes (Fig. 4.12 A, B). In Fig. 4.12 A, the liposome can be seen bound all over the pollen tube and possibly inside some pollen tubes (Fig. 4.12 B). It is clear that the liposome did not stick to the pollen tube tip; it is thought that the reason was because the pollen was growing all the time. In the control pollen was incubated in the presence of 6-CF and stained with DAPI to demonstrated membrane integrity. The fluorescence was observed around the pollen grain and in the region near the pollen tube tip (Fig. 4.12 C).

4.11.3 Effect of boric acid on pollen germinated with liposome

Boric acid is essential for pollen germination (see Chapter 1; section 1.7.4), but it was thought to inhibit liposome integrity. Liposomes were incubated with pollen and different concentrations of boric acid (0, 1, 10, 50, 100, 300, 500, gm/l). In the media with no boric acid or 1 mg/l, the germination was poor, but with 10 mg/l or

Fig. 4.12 Interaction of liposomes with pollen in vitro.

- (A)(B) Tobacco pollen after 1 hr incubation with liposomes containing DNA stained with DAPI. Note that liposomes bind all over the pollen tube except the pollen tube tip (x 1150).
- (C) Pollen incubated in the presence of 6-CF and stained with DAPI to demonstrate membrane integrity. Note that staining observed in the pollen grain and in the tip, after 1 hr incubation (x 1150).
- (D)(E) Location of dissolved herring sperm DNA following interaction with pollen tube. Note that the whole tube fluoresced when the pollen tube was incubated without DNase treatment (D), and just the top part of the tube fluoresced when the pollen was incubated with DNA and followed DNase treatment (E). They were stained with DAPI, after 1 hr incubation (x 112).
- (F)(G) Positively charged liposomes were attracted to the tube and rebelled by the pollen grain after 45 min incubation (F; x 450 and G; x 112).

A



B



C



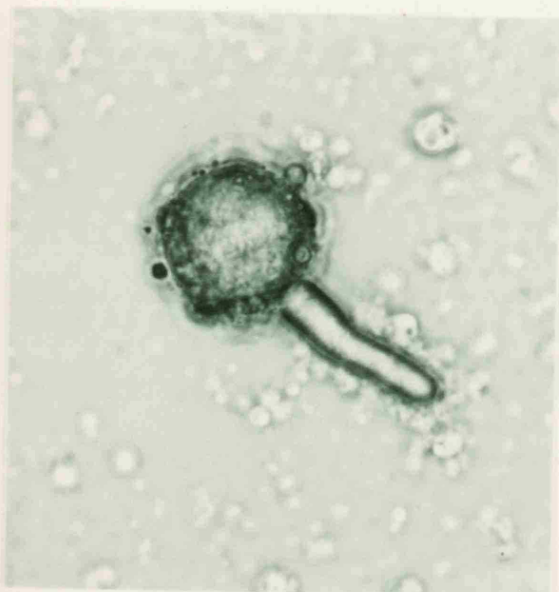
D



E



F



G



between 50 to 500 mg/l, there was no difference (Table 4.9). So, boric acid could be reduced to 10 mg/l, with no harm to either pollen or liposome.

4.11.4 The effect of temperature on pollen germinated with liposome

Liposomes and pollen were grown under different temperatures to find out the appropriate temperature in which both the pollen growth and the liposomes integrity (10, 15, 20, 30, 40°C) was maintained. Between 20 to 30°C was the optimum for tube growth and liposomes or dye uptake; and this result agreed with Ahokas (1987) and Gad, et al., (1988).

4.11.5 The effect of PEG on pollen germinated with liposome

Different concentrations of PEG 6000 were added to the germinating media with liposomes to stimulate liposome binding and uptake. PEG was used widely for liposome uptake by protoplast (see Section 4.2.6).

In this experiment, higher concentrations of PEG 15%, 20%, 25% and 30% inhibited the pollen tube growth possibly because it affected the osmotic balance of the media, but 10% or less allowed a good growth (Table 4.10). Regarding the liposomes binding, in controls (no PEG added) liposomes were observed moving around tubes, avoiding contact and some were observed clustering round the tubes. At 10%, 15% and 25% PEG, the liposome binding to the tubes was high (around 20%). At 15% the tubes became shorter and liposomes were observed on tube tips

Con. of boric acid (mg/ml)	Tube length (μm) average of 25 pollen tubes	Percent germination
0	73	3.5
1	156	22.65
10	299	54.75
50	355	58.9
100	361	59.4
300	362	46
500	331.5	44.5

Table 4.9 The effect of various concentrations of boric acid on pollen germinated in the presence of liposomes.

Con. of PEG %	% germinated pollen which binded to liposomes	percent germination
0	5	69
10	20.8	61.3
15	17.1	59
25	19.9	31

Table 4.10 The effect of various concentrations of PEG on pollen tube germination and binding of liposomes.

and at 25%, the germination was poor, but still the liposomes were observed binding to the short tubes. At 10% PEG the liposomes were seen also in the cytoplasm of the burst tubes, but no indication of whether these liposomes came from the media or from the cytoplasm was obtained. So, 10% PEG is recommended for stimulating at least liposome binding and did not inhibit pollen growth.

4.11.6 The effect of DNase on DNA inside/outside the pollen tube

Pollen was incubated with herring sperm DNA (0.01 mg/ml), then treated with DNase to test if the DNA was taken up by the tube and whether the DNA can be broken inside the tube by the DNase treatment. It was found that when pollen was incubated with DNA without DNase treatment, and the DNA stained with quinacrine then the whole tube fluoresced (Fig. 4.12 D). The pollen which was incubated with DNA followed by treatment with DNase (see Chapter 2; section 2.20.8), only showed quinacrine staining at the top region of the pollen tube (Fig. 4.12 E). This result perhaps indicated that the DNA was taken by the tube and that the DNase did not effect the DNA inside the tube or it may have broken down partly.

4.11.7 The effect of positively charged liposomes on pollen grain and pollen tube development

In this experiment, the charge of the pollen grain and tube was investigated. Positively charged liposomes were formed by using 83 μ l of lipids (egg lecithin: cholesterol:stearylamine 42:12:6 μ M). Liposomes were

attracted to the pollen tubes, but repelled by the pollen grains (Fig. 4.12 F). This result showed that the surface charge of the pollen grain is negative and the pollen tube is positive.

4.11.8 Radiolabelled plasmid-encapsulating liposomes

Plasmid was labelled with ^{35}S (see Chapter 2; section 2.17.1). The radioactive plasmid was incorporated into a suspension of liposomes. 200 μl liposome suspension incubated with the content of one anther of tobacco pollen.

Treatments were done as following: In the first treatment, the pollen was incubated with unlabelled plasmid as a control. The second treatment involved incubating the pollen with labelled plasmid. In the third treatment, pollen was incubated with labelled plasmid for 30 min, then washed 3 times and allowed to continue growing for 1 hr.

There appeared to be more radioactivity around pollen tubes where the liposomes had apparently stuck. It was very difficult to determine whether radioactivity was inside or outside the tube. The radioactivity was rather low, and it is very important that a high concentration of liposomes should be used in the future.

4.12 ELECTROPORATION OF POLLEN TUBES WITH 6-CARBOXYFLUORESCCEIN

A convenient way of monitoring the uptake of molecules into the cytoplasm makes use of the self-quenching fluorescent dye 6-CF (Weinstein et al.,

1977). This chemical is self-quenching at high concentration but fluoresces when diluted, for example, after entry into the cytoplasm. Therefore, this chemical can be used to reveal even small increases in the permeability of the plasma membrane. Tobacco pollen was germinated in 1 ml SM in presence of 6-CF, then was subjected to electroporation (see Chapter 2; section 2.16). The pollen showed good germination indicating that the dye did not inhibit tube development. However, no fluorescence was observed in the pollen tubes.

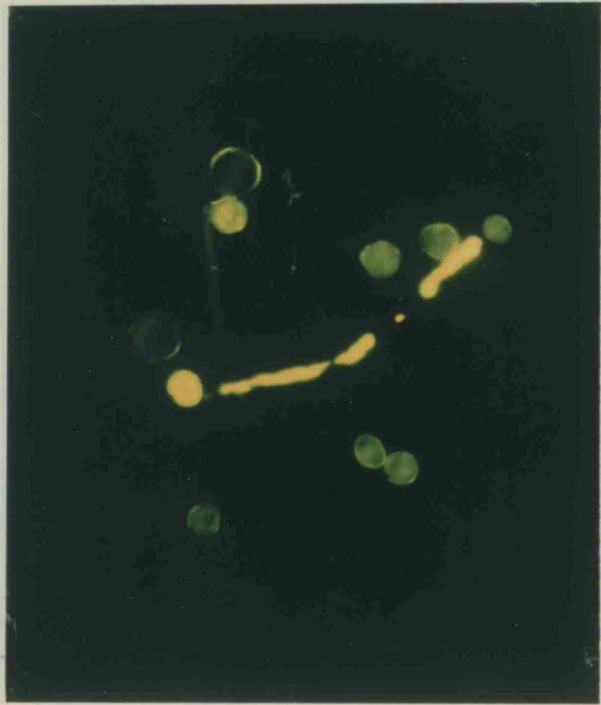
4.13 INCUBATION OF POLLEN TUBES WITH

6-CARBOXYFLUORSCEIN IN PRESENCE OF PEG

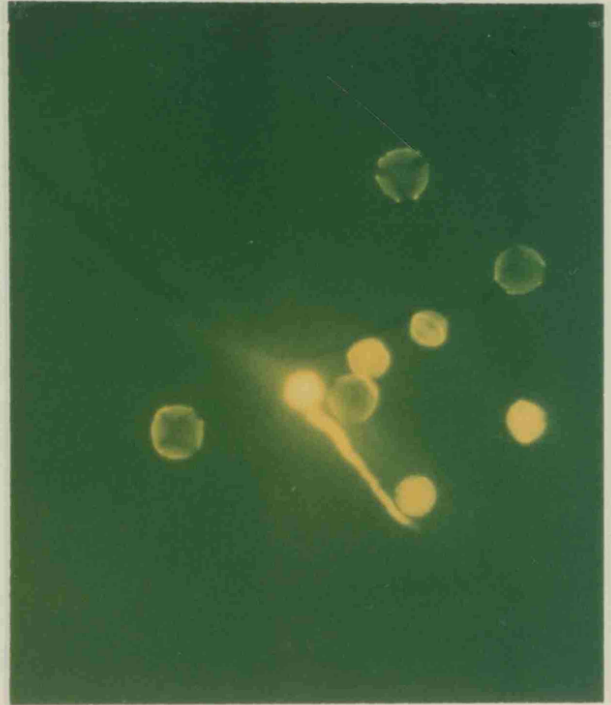
Tobacco pollen tubes were germinated in 1 ml of SM media and 200 μ l of the dye (10 mg/ml 0.1 M NaOH) in presence of different concentrations of PEG (10%, 15%, 25%). The tubes were incubated on a shaker and after 1 hr, the pollen showed good germination and also the dye did not appear to have been taken up by pollen tubes. The pollen tubes were then washed twice by centrifuging the tubes for 1 min, the supernatant was discarded and fresh media was replaced, and then viewed under U.V. microscope. Fluorescence was observed in the pollen tubes (Fig. 4.13 A, B). The percentage of tubes which fluoresced was between 16% to 19% for the concentration between 16% to 25% (Table 4.11), but 10% was recommended because the percent germination was significantly the highest. It was not known whether the 6-CF entered the normal tubes or if some minor damage allowed the leaking of a small amount of the dye into the pollen tube.

Fig.4.13 A,B Fluorescing tobacco pollen tubes following
incubation with 6-CF in the presence of 10%,
PEG, after 1 hr incubation.
Note that some tubes did not fluoresce.

A



B



Con. of PEG %	% germinated pollen which fluoresced	percent germination
0	0	85
10	19.3	61
15	17.6	43
25	16.2	29

Table 4.11 The effect of various concentrations of PEG on pollen germinated with 6-CF.

4.14 SUMMARY

As a prerequisite for transformation, it was shown that the marker genes for transformation would work in pea. Pea was treated with *Agrobacterium*, and a tumour was induced which indicated that the plant can be engineered. Using *Agrobacterium* strains virulent on pea it was then shown that the transformation marker genes were expressed efficiently. Accordingly pea pollen was then attempted to be used as a target for transformation.

A very important part of any transformation system is the selection technique. For tobacco, 600 $\mu\text{g/ml}$ of kanamycin is the optimum concentration to be used for inhibiting tobacco seedling growth. For pea, it was appropriate to explore the use of screening technology. In pollen of transformed tobacco plants the npt-II gene was probably silenced due to the decline of transcription during the early pollen germination period; it is difficult to generalise this result (Bino et al., 1987), and more research can be done with other species.

In this chapter, several methods for introducing plasmid DNA into pollen tubes in vitro were attempted using marker genes which have been used successfully to transform protoplasts (see Section 4.2). Moreover, methods for detecting plasmid DNA also were attempted.

The experiments demonstrated that plasmid DNA can bind to the pollen tubes as detected by labelled liposomes and biotin-labelled DNA. No attempts were made to measure the amount of liposome-encapsulated DNA or dye transferred into recipient pollen tubes. However it was clear that only small amounts of liposomes or biotin-labelled DNA

were bound to, or had possibly entered the pollen tube.

Another method for detecting plasmid is autoradiography and these experiments again did not give a clear result because of background problems; it might be worth repeating the experiments with a more stringent procedure for protecting the slides from radiation, strong electromagnetic forces and light.

The transformation experiments (PEG, incubation, coating pollen with plasmid, electroporation, microprojectile), gave negative results possibly because of problems with nuclease activity (see Chapter 6). High concentrations of PEG affected the pollen germination and fertilisation, 10% PEG or less if possible is recommended. Coating pollen grain with plasmids then polleniation can be attempted with other species such as cereal as the period of pollen germination and fertilisation is short and the distance between the stigma to the ovule is short.

Appendix 1
MSO

Component	Conc. (mg/l)
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440
NH_4NO_3	1650
KNO_3	1900
KI	0.830
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025
KH_2PO_4	170
H_3BO_3	6.2
$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
$\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$	8.6
FeSO_4	27.85
Na_2EDTA	37.25
Glycine	2
Inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Sucrose	30 000.000
pH 7.5 - 5.8	

Agar may be added if required at a concentration of 8 g/l.

CHAPTER 5

5.0 DNA UPTAKE INTO POLLEN TUBES IN VIVO

5.1 INTRODUCTION

Introducing DNA *in vivo* is a promising approach for plant transformation that is not widely used because of a lack of studies defining good target cells within the plant. A few reports dealing with transformation *in vivo* have been published in the last few years which have involved, for example, injecting DNA into space surrounding an inflorescence primordia (e.g. De La Pena et al., 1987); or between the palea and lemma in wheat (Picard et al., 1988); rice (Luo and Wu, 1988); and maize (Otha, 1986); or the injection of DNA into the developing grain at the milk maturity stage (Soyfer, 1980). In this chapter it is proposed to assess the possibility of

introducing foreign DNA into gametes or zygotes during normal fertilisation.

5.1.1 Background

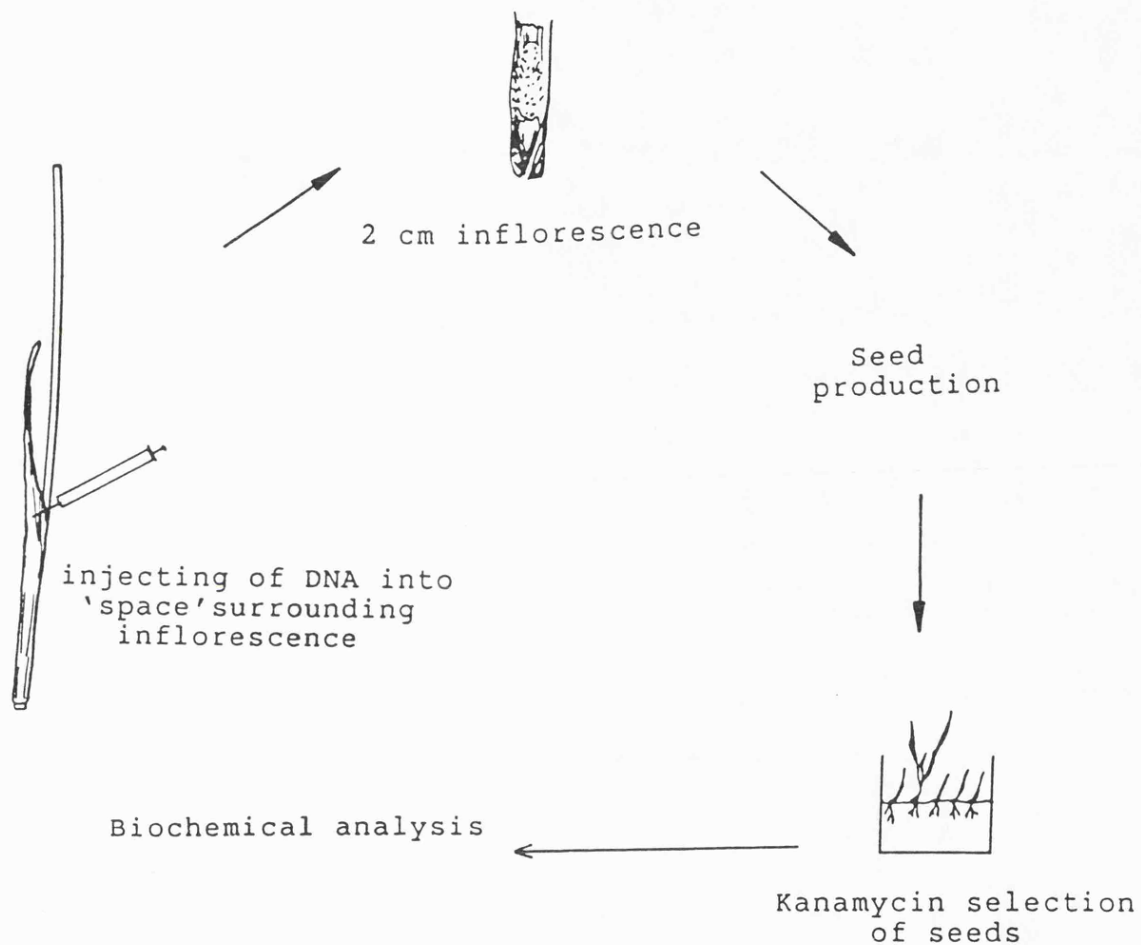
5.1.1.2 Macroinjection

De La Pena et al. (1987) showed that the archesporial cells of rye can take up and integrate exogenously supplied plasmid DNA (pCaMVNEO; see Chapter 2; Fig. 2.1 C) injected into the developing floral tillers (Fig. 5.1). Since the archesporial cells give rise to the gametic tissue, transformed seeds were generated directly following fertilisation. Transformed seeds were selected by germination on kanamycin, and in all, 7 out of 3000 seedlings proved to be antibiotic resistant. This work has yet to be repeated successfully and was the 'state of the art' when the work reported in this section of this thesis was initiated.

5.1.1.3 Pollen grain tubes as a 'pathway' for transformation

Soon after the work described in this chapter was completed, methods for transferring foreign DNA via rice and wheat florets, known as the 'pollen-tube pathway' were reported (Luo and Wu, 1988 and Picard et al., 1988). At the flowering stage, two-thirds to three-quarters of the rice florets were cut, so that the stigma was removed and the style had a severed end (Fig. 5.2 A). 2 - 3 μ l of DNA solution was placed at the cut end of the florets. Up to 20% of the rice seeds from treated florets gave plants that contained the foreign gene, as revealed by Southern-

Fig. 5.1 Rye transformation by 'macroinjection' DNA is injected into the space surrounding 2 cm inflorescence about 14 days before meiosis (from De La Pena et al., 1987).



blot analysis (Luo and Wu, 1988).

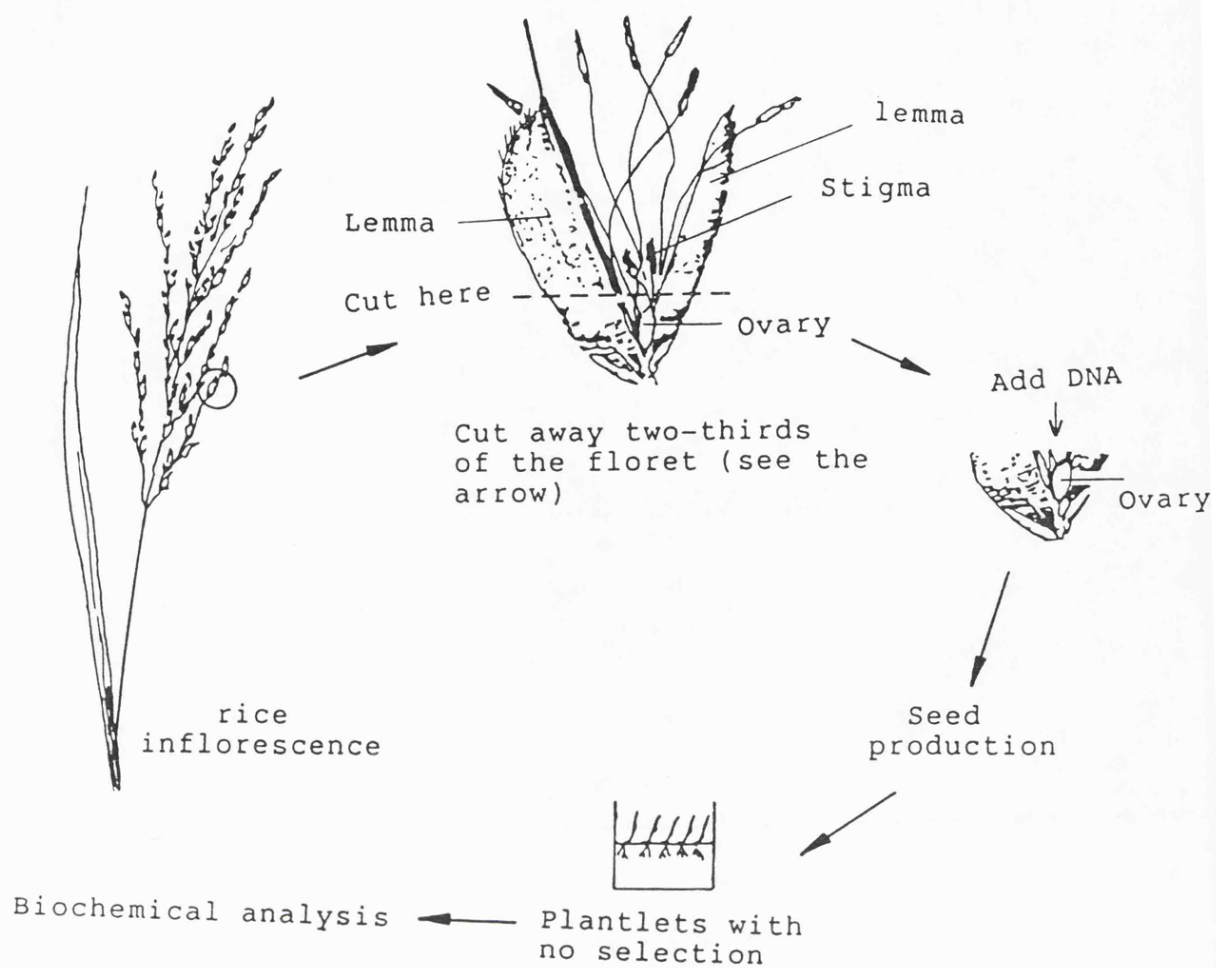
Several varieties of wheat were transformed by Picard et al. (1988). Florets were pollinated by their own pollen or the pollen of another variety. Forty minutes to two hours after pollination, 5 - 10 μ l of a plasmid solution was added into the cavity between the palea and lemma (Fig 5.2 B). 1961 seeds were harvested and germinated on filter paper soaked with a water solution containing 100 mg/l kanamycin. While the great majority of the seedlings bleached at different stages, 19 plants remained normally green and grew fairly well. 1% of these plants contained the foreign genes proved by Southern blot (Picard et al., 1988). Unfortunately nobody has been able to repeat these experiments successfully.

A high efficiency of transfer of genetic characters between distinct maize varieties has been reported by Otha (1986), by self-pollination of recipient female plants (recessive alleles; selfed, kernel have bronzy red aleurone, shrunken endosperm and white waxy starch) with DNA of the donor plant (dominant alleles; selfing, kernels have colourless aleurone, non-shrunken endosperm and yellow, non-waxy starch). DNA was suspended in 0.3 M sucrose to adjust the osmotic pressure and 200 μ l of the solution was applied to each ear. A certain amount of fresh pollen of a recipient plant was taken at the time of anthesis, and mixed with the DNA solution and the pasty pollen/DNA mixture was placed either immediately or about 5 or 10 min later, onto the silk of the same recipient plant. Another method of DNA application was by placing only DNA solution on the silks, and then pollinating as

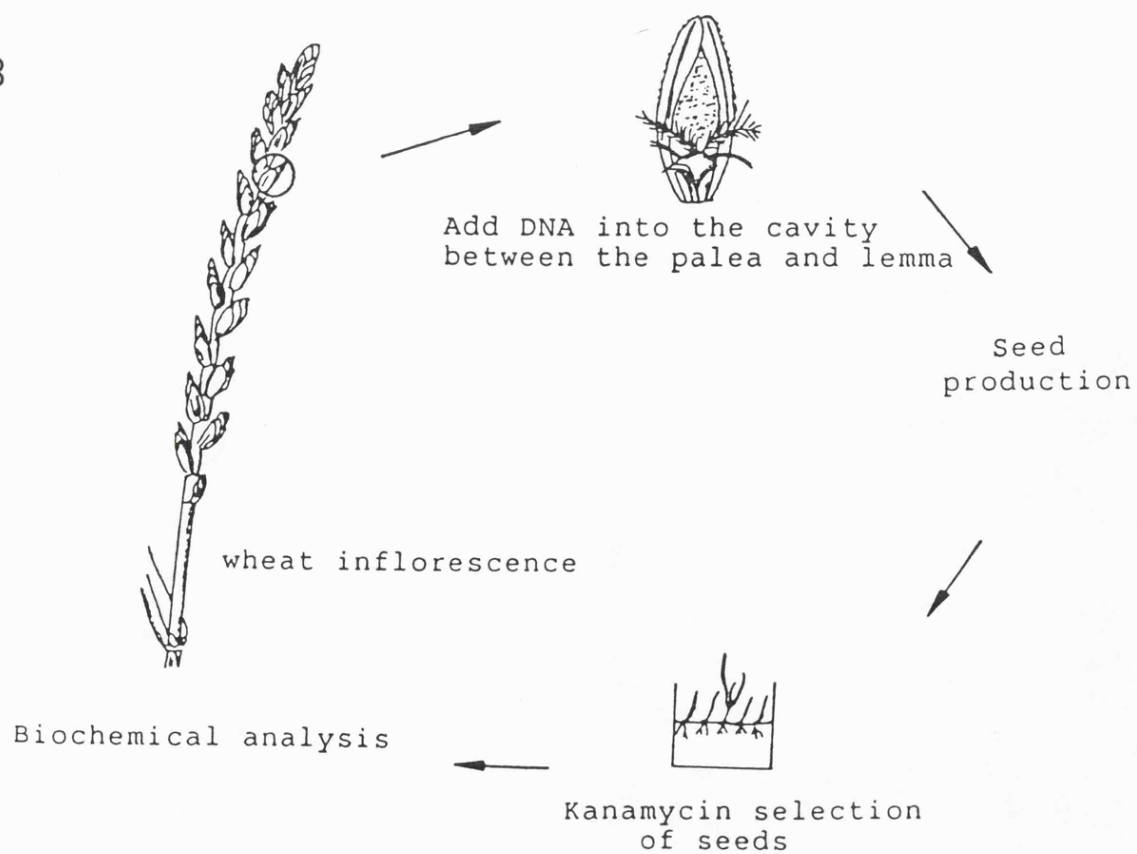
Fig. 5.2 Pollen tube path way for transformation.

- (A) Rice transformation by placing DNA into the region where the stigma was removed and the style had a severed end, then pollination (from Luo and Wu, 1988).
- (B) Wheat transformation by germinating pollen in vivo in presence of vector DNA, then placing DNA into the cavity between the palea and lemma, then pollination (from Picard et al., 1988).

A



B



usual. Self-pollination without DNA was performed as the control. The F₁ hybrid possessed a distinctly different phenotype: colourless aleurone and nonshrunk (round) endosperm with white or faintly yellow nonwaxy starch. The highest frequency of transformed kernels per ear was 9.29%. The most distinct phenotype was colourless aleurone, and shrunk endosperm with white waxy starch; indicating that only a single gene (*I*) had been transformed. Kernels with a colourless aleurone and a waxy starch were obtained, indicating that this particular endosperm had received both *I* and *sh* genes together. The maintenance of transformed characters in the next generation was not as high as in ordinary inheritance through sexual reproduction; the explanation that it may be that the exogenous DNA taken into the embryo was unstable. Clear proof of transformation was not obtained.

5.1.1.4 Injection into immature barley seeds

Injection of exogenous DNA into grains of barley recipient plants at the milk maturity stage has been reported (Soyfer, 1980). DNA from wild-type barley (*Yuzhny* variety; with two-rowed and normal starch) was injected into fertile grains of barley recipient plants (*Waxy* mutant; with six-rowed and without amylase in the starch) at the milk maturity stage. 82 plants that were grown from seeds, showed changed pollen, one in three pollen grains stained black with iodine, while the mutant pollen became brown or red-brown after the staining. In the 2nd generation only 2 plants from the 8 studied preserved

these changes. In the progeny of these two plants, i.e., in the 3rd seed generation after injection 82.1% of plants preserved the transformed characters. The next (4th) generation preserved a transformed phenotype in 89.6% of plants. Also, it was found that a reversion of transformed properties (i.e., normal starch and two-rowed spikes) occurred, in 40% of the 4th generation descendants of one of the plants (Soyfer, 1980).

As indicated above, from these references it is not certain that foreign genes can be taken up *in vivo* to achieve transformed plants reproducibly. The aim of the work reported in this chapter was to attempt to introduce DNA using pollen tubes *in vivo*. It was shown in chapter 4 that DNA can stick to the pollen tube and probably can be taken through the pollen tube tip. So, many methods can be applied *in vivo* and the DNA can be introduced either at pollination, or as the pollen tube grows down the style, or at fertilisation (sperm and egg fusion). DNA can be applied onto the stigma followed pollination or pollen grains can be removed after germination either mechanically or by the use of enzymes. The possibility of infecting mature pollen grains by *Agrobacterium* at the time of pollination or immature pollen grain at early developmental stages also has been investigated. For wheat, as its pollen is not amenable for growth *in vitro* the pollen tube as a carrier of DNA was also assessed as an alternative to *in vitro* transformation.

5.2 POLLINATION BIOLOGY

Temperature and other environmental factors control the opening of the anther. The pollen transfers to the stigma either of the same flower (self-pollination) or to stigmas of flowers of other plants of the species (cross-pollination). For the successful attainment of fertilisation, pollen and stigma must provide not only a suitable chemical milieu for pollen function but also the appropriate physical conditions for attachment and water transfer. A broad distinction can be made between species where the stigma is 'dry' (trinuclate pollen) in the sense that it carries no free-flowing secretion, and those where the stigma surface is 'wet' (binuclate pollen) bearing such a secretion during the receptive period. A wet stigma produces a stigmatic exudate containing free sugar, lipids, phenolic compounds and traces of enzymes (Heslop-Harrison and Shivanna, 1977).

A large number of pollen tubes break during fertilisation and numerous surviving pollen tubes fail to penetrate stigma (De Wet et al., 1986).

5.3 FERTILISATION PROCESS

On the arrival at the ovary (carpel), the pollen tubes travel round intercellular spaces on the inside of the ovary wall until they reach an ovule placenta. On arrival at the embryo sac the tube usually broadens before sperm release, sperm travelling into the central of the embryo sac. At this point, one haploid sperm cell (male gamete) fuses with the haploid egg cell to form the

diploid zygote which will form the embryo in the developing seed, and thus develop into the new sporophyte generation. Fusion occurs through the evagination of the female egg cell and enclosure of the sperm cell, apparently including its cytoplasmic contents. Once enclosed, the sperm cell membrane breaks down to release the nucleus, which lies against the female nucleus. The second haploid sperm cell fuses with the primary endosperm nucleus (polar nuclei), the result is a triploid primary endosperm cell, which develops into the endosperm that acts as nurse tissue (protection and feeding) to the embryo in the seed. The antipodals are at the opposite end of the ovule and undergo endomitosis, to form a block of antipodal tissue of unknown function in the seed. One or both synergids are located near the micropylar end start to disintegrate before accepting the pollen tube and sperms; if only one is involved in sperm transport, the other also soon disappears (Fahn, 1974; Bidwell, 1979; Scagel et al., 1984; Richards, 1986).

5.4 FEATURES RELEVANT TO IN VIVO POLLEN MEDIATED TRANSFORMATION FOR PEA AND TOBACCO

As mentioned in chapter 2, the present study shows that the region of the tobacco stigma receptive to pollen tube penetration is between 1.5 - 2.0 mm diameter and that thousands of pollen grains land on the stigma during pollination and 80% of pollen grains germinate and usually between 1500 - 2000 seeds are set in each ovary. The optimum time for applying germinated pollen is within the first 2 hr because with increasing incubation time the

number of seeds set was decreased. It was observed that the germinating pollen in static culture gave a better yield of seeds than germinating pollen in shaken culture. Between 18 - 215 seeds were obtained from ovaries pollinated with pre-germinated pollen in vitro. Pea stigmas are very small (500 - 1000 μm); less than 1% of the pollen germinate resulting normally between 3 - 6 seeds per ovary. Between 0.4 - 0.6 seeds per pod were obtained from ovaries pollinated with pre-germinated pollen.

5.5 RESULTS

The plasmid DNA used in vivo experiments was supplied in media at the correct osmotic value. The plasmid was kept in Q water and addition of up to 50 μl of plasmid did not effect the germinating media and the pollen grew normally; if the plasmid solution was a larger component than this, the pollen germination was slightly reduced and more sugar was added to the media to keep its osmotic pressure correct. The treated pollen which was applied to the stigmas gave less seeds than the control (79%).

5.5.1 Treating tobacco and pea stigmas with plasmid before pollination

In this experiment, the pollen was placed on the plasmid solution which guarantees that every tube must pass through the plasmid and which might allow plasmid to be taken to the ovule by the tube.

The Q water containing CaMVNEO (300 $\mu\text{g/ml}$; see Chapter 2; Fig. 2.1 C) was placed onto tobacco stigmas and

pea stigmas then freshly dehisced pollen was placed on the drop. In some experiments, SM was added to the plasmid solution to keep the osmotic balance and to avoid burstage of the pollen tubes. For tobacco thousands of seeds were obtained, but when using plasmid in osmotically-balanced media, more seeds were obtained than in just Q water. For pea, using plasmid in Q water, no seeds were obtained due to pollen burstage, but after using plasmid in 50% Q water and 50% BM, seed set was more or less normal.

The tobacco seeds were plated on MSO media (see Appendix 1) containing 600 $\mu\text{g/ml}$ kanamycin. After 2 weeks, all the seedlings bleached and 20 transformed seeds [SR1 (CAB GUS)], used as a control developed as normal. Pea seedlings grown also on MSO media (see Appendix 1) containing 62.5 $\mu\text{g/ml}$ kanamycin, all bleached.

5.5.2 Removal of tobacco pollen grains by pectinase treatment

The elongation of the pollen tube through the style may serve as a way for transferring DNA, especially for species that have short tubes. In this experiment the grains were removed by pectinase (10,000 units). The original idea was to remove the outer pectic coating of the tube by pectinase and the cellulosic middle layer and the inner callosic sheath by other enzymes, in order to give access of the DNA to the tube plasmalemma; however it was found that the pectinase separated the grain from its tube, it may be because high concentration of pectin exists between the grain and the tube.

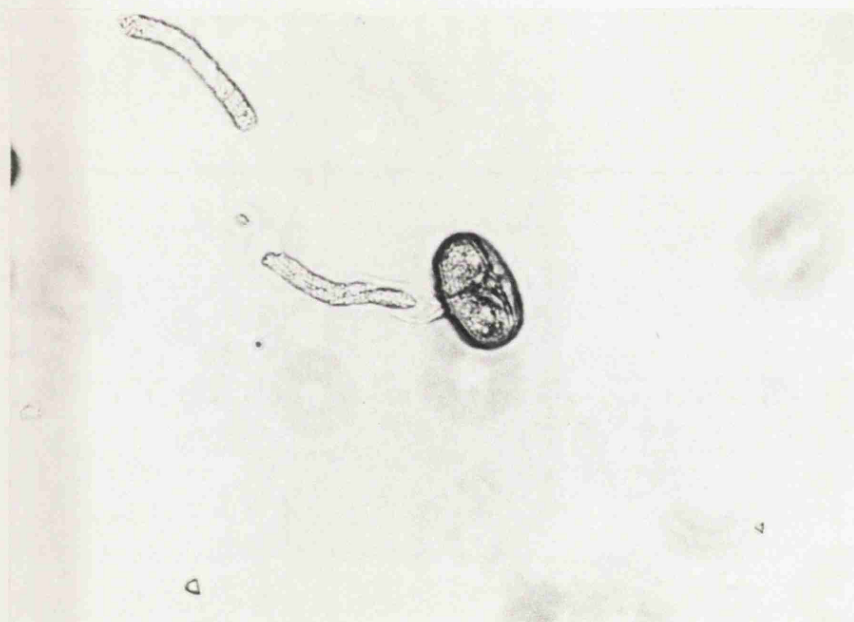
5.5.2.1 Pectinase treatment *in vitro*

Pollen from 6 freshly dehiscent anthers of tobacco was placed in 6 ml SM, the stock was distributed to 6 tubes and incubated for 1 hr. Different concentrations of pectinase were made by adding DH_2O to each tube (1000, 2000, 4000, 6000, 8000 or 10000 units). The pollen germinated normally at the concentration of 1000, 2000 or 4000 units of pectinase. In pollen germinated at 6000 and 8000 units of pectinase, 30% of the tubes were disconnected from their grains and 62% were fragmented at some point of their length (Fig 5.3). The cytoplasm appeared to vacate a small area in certain tubes and this was adjudged to be the probable point of breakage. These fragments were visible and were invariably full of cytoplasm with very little leakage. It is possible that the presence of pectinase, in addition to causing tube cleavage, initiates a pinching of the plasma membrane to encase two bodies of cytoplasm. This may be related to the natural system of laying down of callose plugs. In this work, this may provide an ideal mechanism for introducing plasmid into the cytoplasm of germinated pollen *in vivo* on the stigma.

5.5.2.2 Pectinase treatment *in vivo*

Pollen was applied to emasculated flowers of tobacco followed by pectinase treatment after 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 hr by placing a drop of pectinase (6000 units) onto the stigmas in order to separate the grains from the pollen tubes. Detached grains were washed from the stigmas with germination medium. To demonstrate

Fig. 5.3 Effect of pectinase on germinated pollen of pea.
Note that the tube was in process of disconnection
from the grain after 1 hr incubation. In the same
picture a fragment of tube (x 380).



whether the wash did remove the grain or the pectinase, pollen was applied to emasculated flowers after 1, 2, 4, 6, 7, 8, 12, and 24 hr and then the stigmas were washed off also with medium. The stigmata which were treated with pectinase and treated with media after 1, 2, 3, and 4 hr did not produce seeds, but after 5, 6, 7, 8, 12 and 24 hr, seeds were obtained (Table 5.1). In plants where the stigmas which were treated with medium and washed off also with medium, after 1 hr, no seeds were obtained. After 2, 4, 6, 12 and 24 hr, seeds were obtained (Table 5.1). This indicated that the pectinase can be added only after 5 hr or more in which time the pollen germination and penetration had taken place.

5.5.2.3 Plasmid treatment in vivo

Emasculated tobacco and pea flowers were prepared and at 11.00 am the stigmas were pollinated; after 5 hr (4.00 pm) pectinase solution was placed onto the stigmas and after a further 1 hr, the pectinase was washed off with media. 5 μ l plasmid solution pBI.121 (300 μ g/ml: see Chapter 2; Fig 2.1 A) was placed on each stigma. Also plasmid solution was added to a cut blue Gilson tip and the tip was placed on to the treated stigmas to provide a continuous supply of vector. Seeds were harvested from all treatments and then selected onto 600 μ l/ml kanamycin, but no transformation was achieved.

5.5.3 Removing tobacco and pea pollen grains from the stigmas mechanically

In this experiment, the grains were removed by a

Time (hr)	No. of seed set after pectinase treatment
1	0
2	0
3	0
4	0
5	4
6	140
7	200
8	230
12	500
24	800
Time (hr)	No. of seed set after media treatment
1	0
2	262
4	319
6	509
12	796
24	Approx. 2500

Table 5.1 The effect of pectinase and media treatments on tobacco fertilisation and subsequently the number of seed set.

sharp scalpel blade and, it was hoped as mentioned with pectinase treatment that the plasmid can make its way to the ovule.

Tobacco and pea stigmas were pollinated (at 11.30 am) and after 6 hr (at 5.30 pm) the grains were removed, 10 μ l plasmid was placed on the stigmas. In another experiment, the plasmid was placed into a cut blue Gilson tip for tobacco and a cut yellow Gilson for pea and placed onto the treated stigmas. Thousands of tobacco seeds and 29 pea seeds were obtained. Seeds were selected (see Section 5.5.1), but no transformation was observed for both tobacco and pea.

5.6 POLLEN-MEDIATED TRANSFORMATION OF WHEAT

Pollen germination is very difficult in Gramineae because the pollen contains three cells at anthesis (Trinucleate) and many attempts to germinate wheat pollen failed in liquid media and gave a short tube in semi-liquid media (see Chapter 2; section 2.5.3 ; Fig. 5.4 A, B).

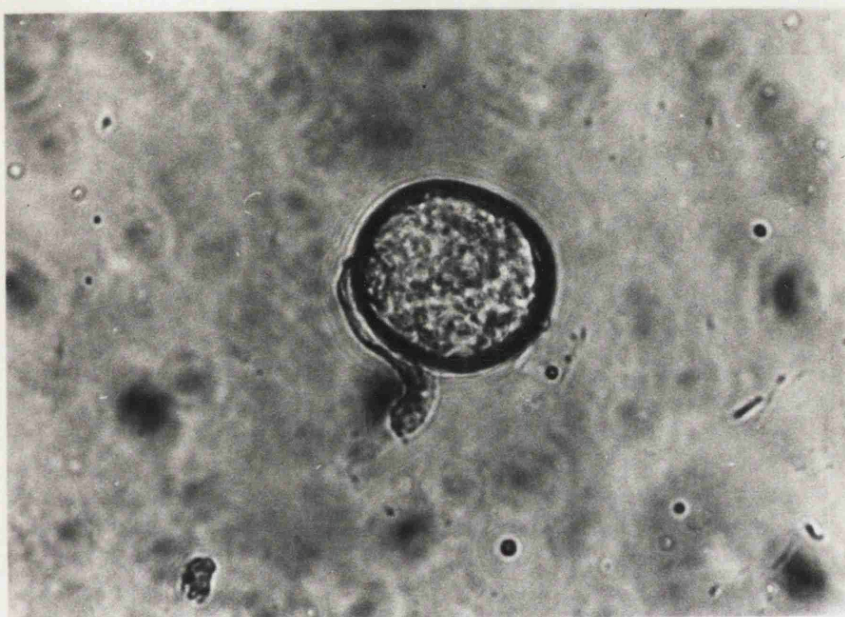
An alternative method is to introduce plasmid via a 'macroinjection' technique. This experiment was designed after De La Pena et al. (1987) who transformed rye by injecting naked plasmid into the surrounding space surrounding inflorescence (see Section 5.1.1.2), showing that the archesporial cells can be a target for transformation.

Plasmid DNA was injected into the natural cavity which exists in a wheat floret between its lemma and palea. The modern bread floret is cleistogamous in

Fig. 5.4

(A) (B) Wheat pollen on semi-liquid media after 4
hr incubation (A; x 380 and B; x 275).

A



B



northern latitudes and so the palea and lemma do not separate during anthesis. Moreover, they constitute a more or less water tight cavity into which plasmids could be easily injected. It was hoped that plasmids applied at various times after anther dehiscence would diffuse into the growing pollen tubes and either incorporate into the genome of one of the sperm cells or else be released with the cytoplasm into the embryo sac during fertilisation. Seeds recovered from the experiment would be germinated and selected for kanamycin resistance, carried by the plasmid.

Seeds of variety "Timmo" were sown, grown under glass house conditions until the spikes florets were about to dehisce. Florets were monitored at 1 - 2 hourly intervals to establish the approximate time of anther dehiscence. Freshly dehisced florets were marked with nail varnish and their position recorded on maps of the fluorescence. 5 - 8 μ g of plasmid (CaMVNEO or CaMVCAT) in 10 μ l of Q water was injected into the lemma-palae cavity (LPC) using a Hamilton syringe at various times (2, 3, 4, 5, 7.5, 8.5, 19, 19.5, 21, 23 or 24 hr) after dehiscence. After each set of treatments, the plants were returned to glass house conditions. After all fertile florets had been treated in this way (or else removed) the plants were returned to the glasshouse and allowed to ripen.

Two sorts of plasmids were used in the experiments: CaMVNEO and CaMVCAT (see Chapter 2; Fig 2.1 C, D)).

A total of 1406 florets were injected with plasmid solution. Of these 1267 were injected with the CaMVCAT

plasmid and 139 with the CaMNEO plasmid (Table 5.2). The 896 seeds that were obtained, were sterilised with 15% Domestos for 20 min and then rinsed 3 times with H₂O. These seeds were separated into batches of 25 seeds and planted onto 60 ml of MSO media (see Appendix 1) supplemented with 60 µg/ml of kanamycin (for CaMVNEO) or 60 µg/ml of chloromphenicol (for CaMVCAT). Control seeds from untreated wheat plants were germinated under the same conditions with and without antibiotics.

All seedlings remained green and grew normally. Moreover, the level of selection used was not sufficiently severe to kill the untransformed seedlings (see the next section). Further, identification of possible transformants proved impractical.

5.6.1 Wheat seedling selection on chloramphenicol and kanamycin

For both chloramphenicol and kanamycin, it was found that the seedlings started bleaching at concentration of 100 µg/ml (Fig. 5.5 A, B and Table 5.3 A, B), which probably, at this concentration the antibiotic resistant plants can survive. Thus in any future work levels of antibiotic must be higher than 100 µg/ml. There was insufficient time to repeat the wheat transformation experiment. Moreover, recently a French group have carried out essentially the same experiment and they obtained transformed wheat (see Section 5.1.1.3); however to date there have been no further data suggesting that these experiments are repeatable.

Code number of Inflorescence	Number of injected florets (CaMVCAT)	Number of seeds set per inflo.
1	41	30
2	44	35
3	8	6
4	77	51
5	64	21
6	64	40
7	38	21
8	45	31
9	25	18
10	55	31
11	57	33
12	59	39
13	34	29
14	34	19
15	62	44
16	53	42
17	48	39
18	32	19
19	34	26
20	56	33
21	67	43
22	52	41
23	39	20
24	46	23
25	27	15
26	57	33
27	49	28
	(CaMVNEO)	
28	36	26
29	38	25
30	26	15
31	39	20

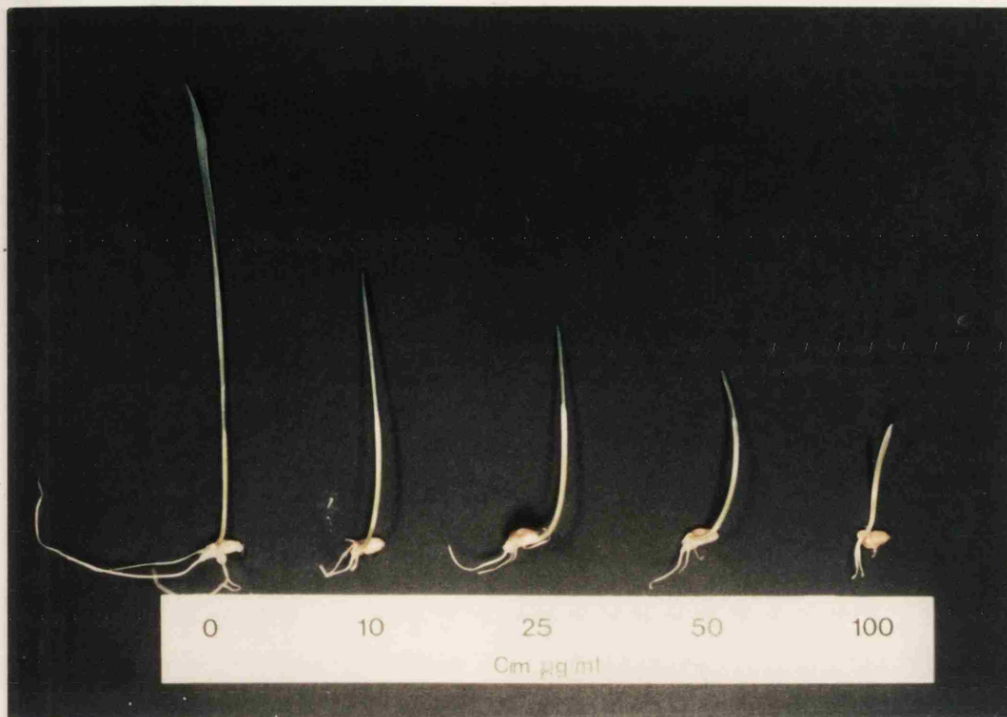
Table 5.2 Seed set after injection of wheat florets with vector DNA.

Fig. 5.5 Effect of chloramphenicol and kanamycin on wheat seedlings.

(A) Chloramphenicol concentrations (see the picture).

(B) Kanamycin concentrations from right to left: 0, 50, 100, 150, 200 $\mu\text{g/ml}$).

A



B



Con. of cm (mg/ml)	Shoot length (centimetre)	Root length (centimetre)
0	16.43	15.30
10	14.16	5.93
25	13.16	5.13
50	11.40	4.1
100	9.93	2.9
150	8.23	2.8
300	3.90	2.06
500	1.66	1.30

Table 5.3 (A) The effect of various concentrations of chloramphenicol on wheat seedling development.

Con. of km (mg/ml)	Shoot length (centimetre)	Root length (centimetre)
0	9.3	8.9
50	5.5	6.1
100	2.3	2.7
150	0	0
300	0	0

Table 5.3 (B) The effect of various concentrations of Km on wheat seedling development.

5.7 ATTEMPTS TO TRANSFORM PEA BY AGROBACTERIUM

5.7.1 DNA uptake by Injection of Agrobacterium into pea flowers

Agrobacterium as a means for transformation has been widely used to transfer foreign genes at wounded sites (see Chapter 4; section 4.1). In this experiment, mature and immature pollen grains were treated with *Agrobacterium* *in vivo*. It was hoped that the *Agrobacterium* could transfer its T-DNA which contained the GUS gene into the generative cell. This experiments was designed after Feldmann and Marks (1987) transformed *Arabidopsis thaliana* seeds by cocultivating with *A. tumefaciens*. Seeds were imbedded 12 hr in a liquid basal medium before a 24 hr exposure to *Agrobacterium*. The problem with the mature pollen grain is the solidity of the wall; it contains sporopollenin which is extremely tough and decay resistant (see Chapter 1; section 1.5.1) and thus it may prevent T-DNA transfer. So, it was hoped that the immature pollen at the tetrad stage which includes four meiosis products from a pollen mother cell may be a better target. The tetrad cell has not formed its full wall and can be amenable to transformation by *Agrobacterium*, resulting in transformed seeds through fertilisation.

Pea flowers (Consort) were treated with *Agrobacterium tumefaciens* (3850 GUS). Mature and immature flowers were treated by stabbing their anthers (each flower contains 10 anthers) with a needle loaded with an overnight culture of *Agrobacteria*, then the keels were filled with 10 x dilution *Agrobacterium*.

Mature and immature flowers were filled with

Agrobacteria with or without opening the keels using a Hamilton syringe (100 μ l). 75 seed were obtained out of a hundred florets treated with plasmid. The seeds were sterilised, then plated on solidified agar MSO media (see Appendix 1) on Petri dishes. The cotyledons and the seeds coat were screened using the GUS histochemical assay (see Chapter 2; section 2.10) but no blue precipitate was observed.

In this experiment, 75 seeds were obtained in spite of the *Agrobacterium* presence in the keel. The wounded anthers were drawn into the *Agrobacterium* solution for at least 4 hr. More studies could be done regarding the susceptibility of pollen grains of different species at different developmental stages to *Agrobacterium* infection.

5.8 SUMMARY

These experiments, which involved either placing DNA on stigmas before pollination or removing the grains either by pectinase or mechanically before adding gave negative results. The first problem was the callose plug; the first callose plug was observed between 4 - 5 hr (250 - 300 μ m tube length) and the second was after 5 - 6 hr (550 - 600 μ m), and this indicated clearly that if the plasmid is applied after more than 4 hr it may stopped by callose plug. Another reason, may be that the fertilisation was disrupted when the DNA was involved or the DNA did not reach the ovule due to the fact that the nucellus forms a tight channel through which the pollen penetrates. This channel could be blocked even more so once the pollen tube has passed the micropyle

(Heberle-Bors et al., 1990). Another problem is the long interval between applying the DNA and fertilisation which is normally between 25 - 30 hr for both tobacco and pea. The purported positive results claimed with wheat (Picard et al., 1988), and rice (Luo and Wu, 1988) were obtained at the same time as the experiments reported in this section. In most cereals, the distance from stigma to ovule and the time between pollination and fertilisation is very short. The pollen grains germinate within 90 sec and reach the ovule within 40 min after pollen deposition (Picard et al., 1988). Under these conditions fertilisation may have occurred while the DNA was still in a good condition.

Attempts to introduce foreign genes into tobacco, pea and wheat using 'pollen tube' pathway gave negative results. For wheat, it may be because of the problem of insufficient selection for the seedlings. In the case of tobacco and pea this may be because the long styles and the plasmid can be either degraded or stopped by the internal plasmamembrane or by the callose plugs (see Chapter 6; section 6.4).

CHAPTER 6

6.0 GENERAL CONCLUSION

In this project, several methods were attempted in order to transform pollen grains or to use pollen tubes as a 'path way' for transformation. As mentioned in Chapter 1 (section 1.8.2) the idea behind the direction of this research is that pollen-mediated gene transfer is not necessarily restricted to the dicotyledons and this system should avoid all the problems associated with tissue culture difficulties such as shoot regeneration, the alteration of the nuclear genome or the chance of genetic chimaery. As a prerequisite for transformation, a reliable in vitro germination system was established for pea and tobacco. Subsequently this pre-germinated pollen

could be used for fertilisation with a reasonable degree of seed set being obtained for both species; this was in spite of the fact that the fertilisation procedure was disrupted in some experiments by adding plasmid (which diluted the germination media) or by removing the pollen grains enzymatically or mechanically from the pollen tubes. The germination and fertilisation results are crucial to the approach and allow transformation experiments to be contemplated.

The feasibility of introducing foreign genes into plants via the transformation of pollen, or using the pollen tube as a pathway, were assessed throughout the thesis. For direct gene transformation, there are many questions to be answered before any attempt to transform pollen grain can be made. For example, can plasmid DNA get into pollen tube quickly before degradation? How can the plasmid travel around inside the pollen tube or to the egg cell? Does the pollen tube have the ability to protect the ovary from any foreign DNA agents? Can the foreign gene integrate with the genome of the generative or the sperm cell? that is, is the pollen grain in a 'competent' stage? In other words, is the pollen grain a good recipient for foreign genes? Attempts to answer some of these questions will be made in the next sections.

6.1 PHYSICAL BARRIERS OF THE POLLEN GRAIN

Introducing foreign genes into the pollen grain/tube can be complicated by many physical barriers. The plasmid has to cross firstly the pollen tube wall and the plasma membrane, then has to travel around under the effect of

the cytoplasmic stream. The plasmid also has to cross the vegetative or sperm cell membrane and also the nuclear membrane which is surrounded by a layer of cytoplasm (see Chapter 1; section 1.5.2). The survival of the naked plasmid until it reaches the nucleus of the generative cell or sperm is thought to be impossible. In protoplasts (see Chapter 1; section 1.4.4) the cell wall is absent and just two membranes have to be crossed and also protoplasts have the ability to regenerate in many species. These advantages have made the protoplast an ideal target for many successful transformation experiments (see Chapter 4; section 4.2). The data presented in this thesis did not show conclusively that it is possible to get DNA inside the generative nuclei.

6.2 COMPETENCE OF POLLEN GRAIN

To transform any cell, it must be in a 'competent state' (see Chapter 1; section 1.1). Regarding the pollen grain, there is transcription during the pollen development and maturation which declines during the early period of pollen tube growth (see Chapter 4; section 4.4.3.2), So far the source of transcription within the pollen grain has not been determined and also the time of mitotic division of generative cell to two sperms is not known. Moreover, no DNA synthesis takes place during pollen tube growth of angiosperms (Heberle-Bors et al., 1990). Pollen has a very active DNA repair system (Jackson, 1987) and it is conceivable that DNA that has entered the nuclei of the vegetative or sperm cell is integrated into their genome is the action of the repair

system (Heberle-Bors et al., 1990). So, lack of full competence in the pollen grain may make the chance of transformation very remote.

6.3 POLLEN TRANSFORMATION IN VITRO VIA POLLINATION

Bombardment of the pollen grains with DNA-loaded microprojectiles recently gave a positive result (transient gene expression) when a pollen-specific promoter was used (Twell et al., 1989). Thus, there is a hope that more pollen-specific promoters fused to screenable or selectable marker genes, may prove to be effective for transformation in the near future. The microprojectile approach overcomes the difficulties of access to mature pollen grains without the DNA being degraded extracellularly. This system is very promising in spite of low number of pollen grains which might be transformed at each 'firing'. Therefore, it is thought that it may be waste of time to apply thousands of pollen grains onto a stigma hoping that one or few of them are transformed.

Liposomes offer an alternative system in which high number of pollen grains can be exposed to liposome which stick very well onto the pollen tube; however still no conclusive transformation data has been reported. One advantage of the microprojectile system is that as the transient expression lasts for 16 - 48 hr, the treated pollen can be applied onto plant stigmas which the gene are still active. Thus, if a foreign gene can be introduced into cereal pollen grain a transformed plant might be obtained as the pollination and fertilisation

take only around 1 hr (Picard et al., 1988). Autoradiography showed the labelled plasmid is certainly outside the pollen tube but it was difficult to prove that any plasmid was inside the pollen tube.

The question of whether a marker gene is able to integrate and express with the genome of the vegetative cell genome or with sperm cell is still to be answered as no reports have been published in this field, but it is thought that obtaining integration and expression is still far from being achieved.

Finally, as there is no conclusive data indicating the stable transformation of pollen grains, it is thought that there is no future for any attempts to transform pollen grains *in vitro* using naked DNA as a result of a lack of competence, physical barriers and nuclease activity. It is suggested that these are the main reasons for not obtaining positive results in the past. Many of the claims of success had no strong molecular evidence for transformation. Overcoming the problems to achieve pollen transformation needs a lot of joint effort between molecular biologists and plant physiologists. The microprojectile approach is probably the best method to continue with in this field as the plasmid DNA can be fired straight to the genome of the vegetative cell or the sperm and the demonstration of transient expression by Twell et al. (1989) is a very good start.

Liposomes also might be used as a vehicle for protection of transforming DNA. It is thought that they still might be a suitable technique at the present time for protecting DNA and much more work could be done to

prove whether the biotin labelled DNA within liposomes is really inside the pollen tube and to see if it binds around the generative cell or the two sperms. Perhaps, liposomes could survive until the time of fertilisation?

6.4 PLANT TRANSFORMATION IN VIVO VIA POLLINATION

Attempts to introduce DNA in vitro with a plant having a long style has a very little potential (the tobacco style length is between 2.5 - 3 cm and pea style length is between 1 - 1.5 cm) because the plasmid has to travel a long way and can be either degraded or stopped by the internal plasmamembrane or by the callose plugs which are formed in the old part of the tube and will stop therefore any plasmid movement to the ovule. So, the plasmid must be applied before callose formation which differs between one plant species to another.

Incubation of *Agrobacterium* with pollen grains can be repeated with other species especially at early developmental stages with *Agrobacterium* containing a specific-pollen promoter to drive a marker gene. Even though it is very important to screen the pollen grains before pollination to make sure that the transformation occurred and also, the possibility of T-DNA transfer to pollen grain can be investigated.

As mentioned in chapter 5, wheat and rice were claimed to have been transformed using a 'pollen tube' pathway but in spite of the work being reported more than two years ago, it still has not been repeated with the original plants or with other species. So, the main question is whether the foreign genes present in the tube

cytoplasm can be transferred into the egg cytoplasm and integrate in the nucleus. The general mechanism of integration of foreign DNA into genomic DNA is not understood (Heberle-Bors et al., 1990). The claim of transferring genes into the rice genome does not present definite molecular proof, however because of the attractiveness of this approach it is still worthy of rigorous testing (Potrykus, 1990).

Placentae-attached ovules (DeVern et al., 1987) as a means for obtaining in vitro fertilisation could be explored by applying a plasmid onto ovules at the time of pollen penetration which can be seen under the microscope.

In conclusion, although all of these transformation experiments reported in this thesis were unsuccessful it is still possible that the plant fertilisation system can be perturbed to allow gene transfer.

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