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**UNIVERSITY OF DURHAM**

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**Studies on *in vitro* manipulation of male and female  
reproductive systems of flowering plants**

*A thesis submitted to  
the Department of Biological Sciences, University of Durham,  
in candidacy for the Degree of Doctor of Philosophy*

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## DEDICATION

*Dedicated to Fatima, Muna, Rasheed and Rana, with love.*

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# ABSTRACT

## STUDIES ON *IN VITRO* MANIPULATION OF MALE AND FEMALE REPRODUCTIVE SYSTEMS OF FLOWERING PLANTS

By Noureldaim Hussein

The overall aim of this research project is to investigate the possibility of using pollen as a vector for transporting extracellular substances to the site of gamete fusion in the embryo sac. Manipulation of plant male and female gametophytes included studies on pollen culture *in vitro*, pollen viability and developmental state, loading of fluorescent probes by plasmolysis/endocytosis and via vascular system, clearance of embryo sacs, ovule culture and the *in vitro* fertilisation and production of genetically uniform lines.

Pollen from *Impatiens glandulifera* cultured under a range of nutrients (sucrose, H<sub>3</sub>BO<sub>4</sub>, Ca, K and Mg), temperature and humidity conditions revealed that 5% sucrose, 100ppm H<sub>3</sub>BO<sub>4</sub> with 100 ppm potassium nitrate, gave longer pollen tubes (463.20 µm after 1 h). Pollen tubes were longer at room temperature; however, they also grew under temperatures down to 4 °C. The effect of humidity levels was also significant, and pollen tube length increased with the increase of relative humidity (RH) over the range 0.0 to 92.0%. Plasmolysis followed by deplasmolysis of pollen gave a non-significant effect on tube growth compared to the control treatment.

Assessment of pollen viability using fluorescein diacetate (FDA) and Calcofluor White M2R (CFW) highlighted some drawbacks on the most widely used technique for assessing pollen viability, the fluorochromatic reaction. Pollen developmental state in *I. glandulifera* was assessed using the Feulgen staining technique. The use of DNA-specific 4,6-diamidino-2-phenyl indole (DAPI) has clearly shown the vegetative and generative nuclei. Pollen tubes were monitored using aniline blue.

When pollen were plasmolysed and deplasmolysed in the presence of 5 mg ml<sup>-1</sup> Lucifer Yellow CH (LY-CH), the fluorescent probe was taken into pollen and the most likely mechanism by which it was taken up was through plasmolysis/endocytosis. The loading up into pollen of FDA by enzymic cleavage and fluorescein by endocytosis is also discussed.

Fluorescein, LY-CH and Calcofluor White M2R were loaded via the vascular system of *Nicotiana tabacum*, *I. glandulifera* and *Brassica napus*. Using fluorescence microscopy, the path of these probes was followed from the pedicel cells up to the ovules.

The demonstration of loading of fluorescent probes into embryo sacs, whether via germinating pollen or via the vascular system, required manipulation of ovaries and clearance of embryo sacs. The fixing and clearing technique revealed, to some extent, embryo elements in *I. glandulifera* and *N. tabacum*. The enzymic maceration technique, however, resulted in the isolation of *I. glandulifera* embryo sacs. The embedding in London Resin White (LR White) technique was used to reveal additional information.

*In vitro* stigmatal pollination of *I. glandulifera* ovaries resulted in pollen tubes penetrating into the ovules. When this was conducted in the presence of Lucifer Yellow CH, *B. napus* pollen tubes were seen carrying the probe and penetrating into the ovule. Fully grown *N. tabacum* plants were obtained from ovaries cultured and pollinated *in vitro*.

Micro-propagation of *I. glandulifera*, *N. tabacum* and *B. napus* in Murishige and Skoog, and Nitsch and Nitsch -based media resulted in plantlets from *B. napus* and *Nicotiana tabacum*. Acclimatisation of the latter, under humid conditions, resulted in fully grown plants.

**THIS THESIS IS ENTIRELY MY OWN WORK  
AND HAS NOT PREVIOUSLY BEEN OFFERED IN  
CANDIDATURE FOR ANY OTHER DEGREE OR  
DIPLOMA**

**Noureldaim Hussein  
August, 1993**

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## LIST OF ABBREVIATIONS

$\mu$ l	microlitre
$\mu$ m	micrometre
$\mu$ m/min	micrometre per minute
6-BAP	6-Benzylaminopurine
BV	Blue-Violet
CFW	Calcofluor White M2R
cm	centimetre
DAA	days after anthesis
DAP	days after pollinating
DAPI	4,6-diamidino-2-phenyl indole
DF	degree of freedom
DNA	deoxyribonucleic acid
ES	embryo sac
FCR	fluorochromatic reaction
FDA	fluorescein diacetate
g	gram
h	hour
l	litre
LR	London Resin
LY-CH	Lucifer Yellow carbohydrazide
M	molar
min	minute
ml	millilitre
mM	millimolar
MS	mean squares
MSO	Murashige and Skoog Basal Medium
NAA	$\alpha$ -naphthaleneacetic acid

nm	nanometer
OCM	ovule culture medium
P	probability
PCM	pollen culture medium
RH	relative humidity
RNA	ribonucleic acid
rpm	revolution per minute
sec	second
SS	sum of squares
StDev	standard deviations
UV	ultra-violet
w/v	weight by volume

# CHAPTER ONE

## INTRODUCTION

### 1.1 Discovery of the basis of sexual reproduction in plants:

The discovery of sexuality in plants can be traced back to the writings of Aristotle and Theophrastus. However, actual observation of the sexual cells was only made possible after the invention of the microscope. Preliminary information on sexuality in plants was provided by Grew (1682) who made the first explicit mention of the stamens as the male organs in the flower. Further scientific discoveries were made by Camerarius (1694) who stated:

"In the plant kingdom, the production of seed which is the most perfect gift of nature and the general means of maintenance of the species, does not take place unless the anthers have previously prepared the young plant material in the ovary".

Kolreuter (1761-1766) fully confirmed the findings by Camerarius and succeeded in producing hybrids in some plant species. In an attempt to assess further the manner in which the pollen influenced the ovule, studies by Amici (1824) and Brongniart (1827) revealed that the interaction between the pollen and the ovule is made possible by pollen tubes.

The origin and development of the ovule was the subject of studies by Schleiden (1837) who published what was known as Schleiden's Theory. He asserted that it is the pollen tube that becomes the embryonal vesicle, and undergoes a number of divisions to form the embryo. This was later refuted by Hofmeister (1849) who rejected Schleiden's theory and showed that the embryo originated from a pre-existing cell in the embryo sac (ES) and not from the pollen tube. Important advances were made by Strasburger (1884) who discovered the actual process of syngamy, or the fusion of the male and female gametes. He showed that one of the two male nuclei



discharged by the pollen tube fuses with the nucleus of the egg. Nawaschin (1898) showed that in angiosperms both male gametes are concerned in fertilisation.

Prior to 1891 it was believed that the pollen tube enters the ovule through the micropyle; Treub in the same year reported pollen penetration through the chalaza. The former is the usual condition and is known as porogamy and the latter is known as chalazogamy. However, there are several modifications depending on the structure of the gametophyte. In *Acacia*, for instance, a micropyle does not exist at the time of fertilisation (Newman, 1934). In other instances, like in *Philadelphus*, *Utricularia*, *Vandellia*, and *Torenia* the ES protrudes out of the micropyle so that the pollen tube comes in direct contact with it (Maheshwari, 1950). Chalazmogamy was also reported in *Rhus* (Grimm, 1912), *Circaeaster* (Junell, 1931a), and a few other genera.

It was Nawaschin who discovered double fertilisation in 1889; the fusion of the male gamete with the egg was regarded as generative fertilisation, while the fusion of polar nuclei with the second male nucleus was called vegetative fertilisation (Strasburger, 1900).

## **1.2 The structure and function of the female gametophyte of flowering plants:**

The megasporangium or ovule consists of the nucellus and, ordinarily, one or two integuments. A third integument is present in some species (e.g. *Euonymus sp.*), which later develops into the aril, an organ concerned with animal dispersal of seeds.

The megaspore mother cell forms a tetrad of four cells through a meiotic division. Different views have been presented with respect to the functioning megaspore. Maheshwari (1950) stated that it is normally the chalazal megaspore which gives rise to the ES. Other reports suggest that it is micropylar megaspore (Feherlind, 1945), or the third megaspore from the micropylar end (Mauritzon, 1934) which gives rise to the ES. There may be variation between taxonomic groups.

The female gametophyte, the ES, typically contains an egg cell, a central cell, synergids flanking the egg cell and variable numbers of antipodal cells, which represent a greatly reduced female gametophyte.

Although both the male and female gametes are intimately involved in plant fertilisation, other cells do apparently play an essential supporting role. An important role attributed to the synergids is that of absorption, synthesis and transport of nutrients. They also play a major nutritional role (Pritchard, 1964; Schultz and Jensen, 1968). However, some authors do not consider the synergids to be involved in the nutritional mechanism (Mogensen, 1972). They also provide the pollen tube and its sperm cells with access to the ES and subsequent dissemination of the sperms to the egg and the central cell. There is good evidence that there is a chemotactic relationship between the pollen tube and the synergids; one of the two synergids begins to degenerate soon after pollination, while the pollen tube is still travelling down the style, and this degenerate synergid forms the point of entry for the pollen tube into the ES.

The central cell remains chiefly engaged in storage of food reserves and after fertilisation its two nuclei unite with a male gamete to form the triploid endosperm. The antipodal cells play no further part in development once the ES has formed.

### **1.3 Reproductive systems of higher plants:**

Sexuality was described by Fryxell (1957) as conferring on plants a "strong selective advantage" which provides the opportunity for genetic recombination and thus variations in populations. This in turn confers evolutionary plasticity in the face of changing environmental conditions.

The control of sexual reproduction, and thus recombination, has long been a major objective for experimental botanists and plant breeders.

It is worth mentioning at this stage, the distribution of organelles during and after fertilisation. It was reported that after penetrating the wall of the ES, the pollen tube may either pass the egg and one synergid as in *Fagopyrum* (Mahony, 1935), or between the ES and a synergid as in *Cardiospermum* (Kadry, 1946), or directly into a synergid as in *Oxalis* (Krupko, 1944). The events that take place during the fertilisation process in angiosperms are illustrated in Figure (1).

Three different classes of maternal effects in plants has been distinguished by Roach and Wulff (1987). These include cytoplasmic genetic, endosperm nuclear, and maternal phenotypic effects. Organelles such as plastids and mitochondria, for instance, are transferred from the maternal plant to the offspring during ovule formation and development. The endosperm nuclear effect results from the two nuclei it contributes to the 3N endosperm. Phenotypic effects are due to the fact that the tissues surrounding the developing embryo and endosperm are all maternal, and they are important determinants of seed dormancy, dispersal, and germination traits.

In addition to the nuclear zygotic contribution, the parental plant does also contribute to the phenotype of the offspring, e.g. seed size. Male cytoplasm can also influence the hereditary expression of yield characters in plant progeny (Fleming, 1975).

#### **1.4 The structure and function of plant gametes:**

The essential function of the male gametophyte, the pollen grain and pollen tube, is to deliver two male gametes into the ES. The pollen grain consists of a protective wall composed of the exine and intine, enclosing a vegetative and a generative cell.

Many more pollen grains are processed than are necessary for fertilisation, the overproduction of male material have a function with respect to other plants and an

impact on human beings as well (Linskens, 1992). For instance, excess pollen grains become a substrate for the microflora of the soil (e.g. *Rhizophidium*), and an ecological niche on the leaf surfaces of plants. Pollen is a principal source of food for many insects. Besides the direct effect of pollen on human health, it can also be used as an accessory food, in addition to the vital role in human civilisation.

The pollen walls govern water movement in and out of the grain. The layers of pollen wall, as described by Erdtman (1966), consist of the exine and the intine. The former layer which is divided into sexine (outer) and nexine (inner) is sporopollenin; the latter is pectocellulosic. Variations between different plants occur due to the relative thickness of the various components.

There may be one or two sperm nuclei (male gametes) present, depending on whether the generative cell has undergone its second mitotic division. In binucleate pollen this division is delayed until the generative cell enters the pollen tube. Brewbaker (1967) reports that 68% of the angiosperm families shed pollen in the binucleate condition (generative cell divided at the times of anthesis) and the remaining 32% shed pollen in the trinucleate condition. However, both types of pollen grains have been reported in the same plant (West, 1930; Junell, 1931a; Poddubnaja-Arnoldi, 1936), probably due to environmental conditions.

There is a strong correlation between number of male gametes in the pollen grain and self-incompatibility system; plants with binucleate pollen generally exhibit gametophytic incompatibility systems and trinucleate species usually have sporophytic systems (Brewbaker, 1967).

The sperm cells are enclosed by both their own plasma membrane and by the inner plasma membrane of the vegetative cell. The male germ unit and the vegetative nucleus constitute the functional unit of male gamete transmission (Dumas *et al.*, 1984).

The various nuclei were reported to be different, both structurally and functionally, as revealed by their staining reactions (LaFountain and Mascarenhas, 1972; Coleman and Goff, 1985; Hough *et al.*, 1985). The male germ cells are initially spindle-shaped and become spherical soon after isolation (Russell and Cass, 1981).

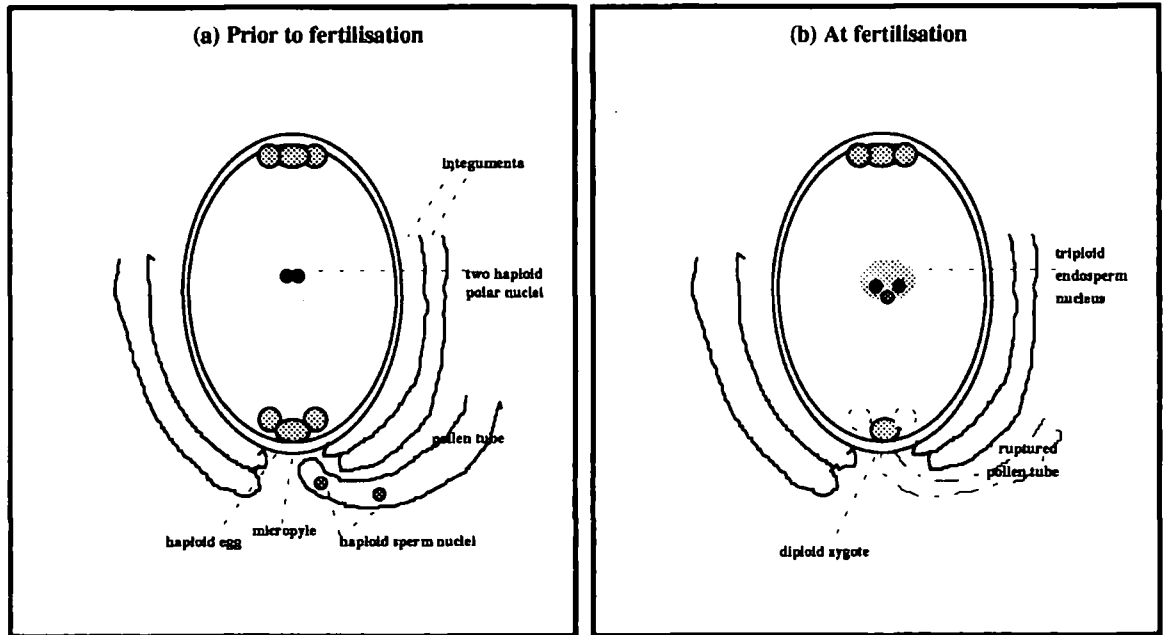
Viable and mature pollen are reported to possess nonvacuolate, granular cytoplasm with many plastids, mitochondria, Golgi-derived vesicles, lipid droplets, and a large population of quiescent dictyosomes (Larson, 1965). Starch grains are also commonly present in some species, such as *Zea mays*.

## **1.5 Pollen germination and tube growth:**

When pollen grains germinate, the pollen-tube wall is synthesised by the carbohydrates and lipid reserves of the grain, supplemented by metabolites taken up from the stigma and style during penetration towards the ES. They consistently show a three-layered structure consisting of an outer pectic coating, cellulosic middle layer and an inner callosic sheath (Heslop-Harrison, 1979).

The pollen tubes serve an important function as a conduit through which the sperm cells move down the style.

**Figure (1): The events that take place during the fertilisation process in angiosperms**



## 1.6 Advances in understanding gained from culturing animal eggs *in vitro*:

The understanding of early zygote development in animals has advanced rapidly, as a direct result of the fact that *in vitro* fertilisation of female gametes can be achieved with relative ease. The fertilisation process consists of the penetration by the motile spermatozoon into the stationary egg cell, and the subsequent union of the two gametes. However, this process is much easier to observe in the gametes of animals such as amphibians, in which external fertilisation takes place. In plants, the fertilisation event takes place deep in the ovule, inside the protective carpel; because of this inaccessibility the precise details of the fertilisation mechanism and early embryo development have still to be described.

*In vitro* fertilisation techniques have allowed rapid advances to be made in the understanding of zygote formation and embryo development in animals. Genetically identical adult vertebrates were first produced by transplanting nuclei from an embryo into a number of enucleated recipient eggs of *Xenopus* (Gurdon, 1961) and in other experiments by transplanting nuclei from one species into egg cytoplasm of another.

In insects, Illmensee and Mahowald (1974) have succeeded in transferring anucleate pole plasm from wild-type *Drosophila* eggs into the anterior pole of genetically marked eggs, prior to cellularization. Nuclear transplantation has also been accomplished in mice by removing the two pronuclei of the zygote and replacing them with pronuclei from other zygotes (McCrath and Solter, 1983).

*In vitro* fertilisation techniques are now routine in livestock breeding and in the restoration of fertility in humans. Techniques has been developed, for the first time, to bring about specific changes to the genetic make-up of farm animals (Wilmut *et al.*, 1988). They made a hybrid gene comprising the protein-coding sequence of

human factor IX and regulatory DNA sequences from a sheep milk protein gene. The gene was then introduced into sheep by direct injection into a pronucleus.

Despite the fact that survival proportion of injected eggs that resulted in transgenic animals is rather low, in mice 2 to 5 per cent, and in farm animals around 1 per cent, this is considered to be enough. When the transgene is taken up by a fertilised egg it becomes incorporated in every cell of the animal. Regulation of transgene expression, however, has been achieved in some instances, by transferring the gene along with neighbouring sequence

### **1.7 The potential value of manipulating plant gametes *in vitro*:**

The potential scientific rewards from *in vitro* manipulation of plant gametes, as compared to *in vivo* observation, are enormous. It would allow the understanding of the later part of the development of the gamete and early zygote development, allow the introduction of xenobiotics which could be used to regulate or modify development, facilitate the production of transgenic plants, via nuclear transplants into pollen tubes or the introduction of foreign DNA, and allow the control and manipulation of the chemical and physical environment for gamete and developing zygote.

Practical, commercial benefits might also be considerable. *In vitro* fertilisation might provide a means of circumventing incompatibility mechanisms that operate within the stigma and style. This in turn would allow new interspecific crosses to be made, broadening the genetic base of crop plants.

Recent evidence has indicated that a large proportion of the microgametophyte genome is transcribed and translated during pollen development, and also expressed during the sporophytic stage of the life cycle (Tanksley, *et al.*, 1981; Willing and Mascarenhas, 1984). It was reported that at least 64% of the pollen



mRNA population of *Tradescantia paludosa* and *Zea mays* was also expressed in shoot tissue (Willing and Mascarenhas, 1984). A similar percentage of genetic overlap between sporophytic and gametophytic phases was also reported in *Lycopersicon esculentum* and *Zea mays* (Tanksley *et al.*, 1981; Sari Gorla *et al.*, 1986).

*In vitro* manipulation of plant male gametes has significantly contributed to the understanding of the basic concepts of fertilisation and the role of sperm cells in this process. Various techniques have been developed to obtain isolated sperm cells (Russell, 1986; Zhou *et al.*, 1986). These techniques include osmotic shock, pH change-induced shock to the exine, or physical grinding.

The transfer of exogenous DNA into pollen as studied by De Wet *et al.* (1984), was conducted by the soaking of pollen with various nucleic acid preparations. They have suggested that germinating pollen tubes can take up exogenous DNA. De Wet *et al.* (1985) reported the absorption of DNA through the pollen tube of maize (*Zea mays* L.) and its incorporation into the genome of the zygote during fertilisation.

By contrast with animals, fertilisation in plants, both *in vivo* and *in vitro*, does not only involve the individual gametes. Other cells are intimately involved, as well, by allowing the sperm and egg cells to gain access to one another. The sperm cells, for instance, are completely enclosed within the cytoplasm of the vegetative cell, and they migrate within the pollen tube. The difference between plant and animal life cycles are shown in Figure (2)

Preliminary results obtained by Hopher *et al.* (1985) suggest the feasibility of the insertion of foreign DNA into the pollen tube. Microinjection of transformed sperm cells may indeed be feasible as reported by Bino and Stephenson (1988). Soyfer (1980) injected DNA into grains of barley at the milk maturity stage in order to facilitate the DNA penetration into embryogenic cells. He concluded that the exogenous DNA had induced changes in some morphological patterns. However,

while the authors mentioned have obtained positive results, negative results have been reported (Coe and Sarkar, 1966; Carlson, 1972).

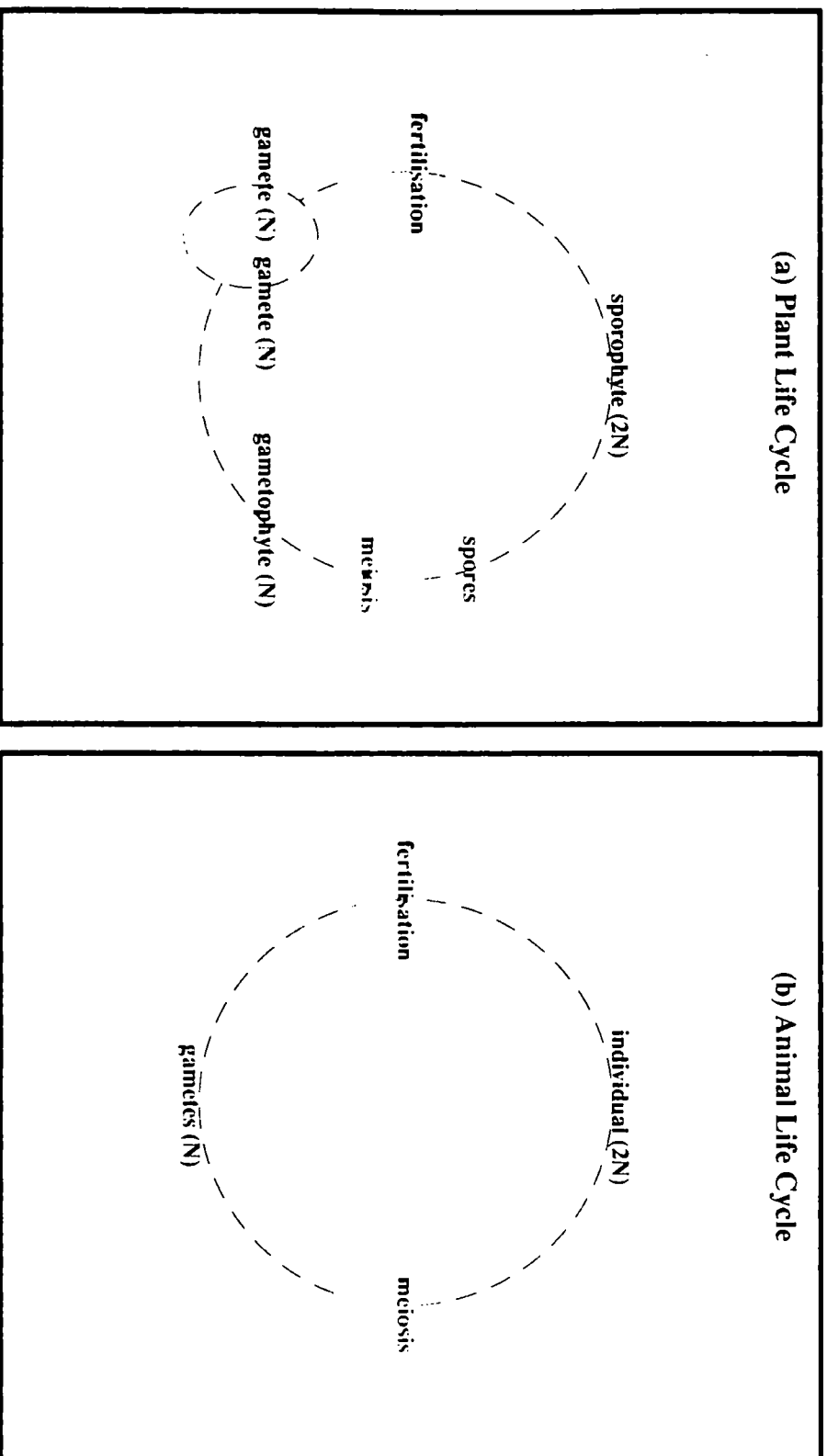
Various techniques have been developed to obtain egg cells under viable conditions. These techniques include micro-dissection of ovaries and ovules, enzymic degradation of cell walls, and squashing. The potential payoff of manipulating female gametes allows for the understanding of the early part of zygote development and opens up various new prospects which could not be achieved *in vivo*: observation of fertilisation processes in the ES, studies of the mechanism of gamete recognition, adhesion and fusion and the insertion of genetic material.

Zhou *et al.* (1983) introduced exogenous DNA into cotton embryos using a combination of injection and transformation. They concluded that the DNA transformed the embryos, by entering the ovule, following the pollen tube path. Soyfer (1980) induced normal starch production among offspring of a waxy-mutant of *Hordeum vulgare* L. (barley) when ovaries were microinjected with DNA of a non-waxy barley genotype. Results obtained by Ohta (1986) revealed that exogenous DNA in the DNA-applied self pollination was transferred through pollen into the ES, and therefore into endosperm nuclei.

## **1.8 The practical difficulties of manipulating plant gametes *in vivo*:**

Many examples of pollen/stigma or pollen/style rejection responses do represent a practical difficulty, holding back successful *in vivo* fertilisation. The interaction between pollen and stigma, as described by Chasan (1992), normally dictate whether pollen tube growth will occur and whether pollination will be successful.

Figure (2) The difference between plant and animal life cycles :





The main difficulty of manipulating plant male gametes lies in the inaccessibility of pollen in the anther. Another difficulty is imposed by the pollen exine. This layer consists of enzyme-resistant sporopollenin and is considered the main barrier against isolation of pollen protoplasts (Yang and Zhou, 1992). However, recent studies (Zhou, 1989a) indicated the feasibility of overcoming this difficulty by hydrating pollen in a medium containing enzymes such as cellulase and pectinase.

The complexity of ovule tissues has been the main barrier hindering the understanding of ES biology as compared with its male counterpart, the pollen. Despite the fact that much concern is being given for the isolation of fixed and viable ESs (Bradley, 1948; Forbes, 1960; Murtey *et al.*, 1979; Zhou and Yang, 1985; Huang and Russell, 1989), ovule inaccessibility remains a practical difficulty of manipulating female gametes *in vivo*.

In order to circumvent such practical difficulties, one option is to load xenobiotics into plant transport system (xylem and phloem) supplying reproductive tissues.

### **1.9 The practical difficulties of manipulating plant gametes *in vitro*:**

The most widely used method of the culture of pollen *in vitro* has been the hanging drop technique (Vasil, 1960). The technique allows microscopic examination and the cultures are easy to store. Where constant microscopic examinations are not anticipated, cultures of large quantities of pollen grains can be maintained in test tubes or flasks with liquid media on a shaker or with the help of bubbled air (Mascarenhas, 1966; Roggen and Stanley, 1969).

Pollen protoplasts have been induced to divide *in vitro* (Zhou, 1989b) and develop small embryogenic masses of cells. This provides experimental evidence that they can be induced to undergo divisions that may lead to plant regeneration. Wu and

Zhou (1990) cultured isolated generative cell of *Hemerocallis minor* in an agarose medium nursed with anthers of the same species. Their findings indicate that generative cells are capable of surviving and undergoing limited development under *in vitro* conditions.

Simple as well as relatively complex nutrient media have been used for the culture of pollen. The simplest of these is a sucrose/boric acid medium which has been used extensively (Vasil, 1960; Brewbaker and Kwack, 1963). More complex media are supplemented with calcium nitrate and potassium nitrate (Dickinson, 1967), calcium nitrate and magnesium sulphate (Kwack, 1967), or calcium chloride, potassium phosphate and yeast extract (Mascarenhas, 1966). In all these different combinations of nutrient media, the pH of the nutrient medium varied from 5.2 to 6.8.

Sucrose is probably the most commonly used source of carbon and energy for pollen growth. However, lactose, dextrose, raffinose and several other sugars and sugar derivatives have also been found useful, while fructose, mannose, and mannitol are most unsatisfactory. As Vasil (1960) reported on his study of Cucurbitaceae species, the optimal germination and maximal length of pollen tubes was obtained under 7.5-20% sucrose. Beyond these concentrations, bursting of pollen, as well as germination are markedly reduced. Other sugar derivatives, like mannitol, gave no pollen germination.

The stimulatory effect of boron on pollen germination and pollen tube growth was first discovered by Schmucker (1932). The role of boron as an essential element for the normal growth and development of higher plants was confirmed by the work of many authors cited by Vasil (1987). Most commonly used forms of the element are boric acid, borax, and butyl borate.

Other chemical substances reported to improve pollen germination and pollen tube growth, as mentioned earlier, include calcium, potassium, magnesium and others.

However, the effect of sucrose and boric acid far surpasses the effect of any other chemical substance or hormone so far used in pollen culture.

The optimal range of temperature that brings about maximum pollen germination and pollen tube length *in vitro* varies between 20 °C and 30 °C. Temperatures up to 45 °C in combination with high relative humidity, were reported to affect pollen vigour more drastically (Shivanna and Cresti, 1989; Shivanna *et al.*, 1991). Pollen treated at 70 °C for 4-24 hr showed no germination at all (Rao *et al.*, 1992).

It has long been appreciated that pollen rehydration is an essential prerequisite for reactivating of pollen as successful attainment of fertilisation requires close physiological coadaptation between pollen and stigma. In a study by Shivanna and Heslop-Harrison (1981), maximum pollen germinability was attained after an exposure of pollen from sedge species to an atmosphere with 95% relative humidity. However, the same study showed little or no effect of prehydration on germinability of pollen from *Cytisus* species.

In a study by Wilms (1981), a decreasing osmotic value was found when descending from the top to the base of the style. Pollen tube growth *in vitro* continues when the medium, in which the growth occurs, shows a gradual decrease of molarity (c.f. Wilms and Keijzer, 1985). A more humid surrounding increases the water content outside the pollen tube and lowers the concentration of the growth medium.

*In vitro* culture of pollen grains was reported to be influenced by a grouping or population effect. Dense and large populations of pollen germinate better and form longer pollen tubes than pollen germinated in small and evenly distributed population in the same medium. Such effect has been noted in a wide variety of species (Brink, 1924; Vasil, 1960; Brewbaker and Kwack, 1963).

The most attractive goal in plant reproductive cell manipulation, however formidable, is female cell culture and intergametic fusion. Egg culture had been tried for many years without success and the main difficulty lies in handling the female cells rather than the male ones (Yang and Zhou, 1992). In a recent advance, Kranz et al (1990) were able to achieve artificially produced zygotes by fusing isolated sperm cell with an isolated egg cell using electroporation.

### **1.10 Uptake of xenobiotics by germinating pollen:**

The examination of the nuclear state during microsporogenesis in mature pollen grains and in germinating pollen tubes is a useful tool to highlight the behaviour and migration of the pollen nuclei during the fertilisation process. The use of xenobiotics has tremendously contributed towards the understanding of pollen nuclear events (Hough, *et al.*, 1985; Coleman and Goff, 1985; Vergne, *et al.*, 1987). Methods for tracing pollen nuclei in the ovule and ovary using the DNA-specific fluorochrome DAPI has been successful with species like *Lilium longiflorum* (Keijzer, *et al.*, 1988) and *Gasteria verrucosa* (Willemse and Keijzer, 1990).

Attempts to genetically transform pollen relied upon the transfer of exogenous DNA into germinated or ungerminated pollen (Hess *et al.* 1974; Hess, 1978) as cited by Hepher *et al.* (1985). Another technique, microinjection, has proved reliable particularly in zoological research (Capecchi, 1980) but Hepher *et al.* (1985) mentioned the limitations brought about by the cellulosic nature of plant cell wall. Due to this limitation, most attempts have centred upon injection into protoplasts.



The potential value of allowing germinating pollen to take up xenobiotics, and deliver these to the ES via the normal process of sexual fusion, is manifold. Xenobiotics could easily be traced microscopically. Another value is that the technique by-passes limitations exerted by the cellulosic nature of the plant cell wall, and avoids problems inherent in regenerating plants from transformed protoplasts. The egg of higher plants is difficult to reach with microinjection (De Wet *et al.* (1985), which necessitates development of new techniques to deliver biologically active material to the ES. The technique, if successful, would offer a valuable means of experimentally manipulating early embryogenesis without having to go through a complex of gamete isolation and/or microinjection techniques.

This task could be achieved by loading molecules into cells endocytically, or by molecules that are transported across the cell membrane and then enzymically cleaved inside cell.

### **1.11 Loading fluorochromes into plant reproductive cells via the vascular system:**

Endocytosis may be defined as the uptake of extracellular substances into the cell, achieved through invagination and then vesiculation of the plasmalemma. Two types of endocytosis are distinguished by Robinson and Hillmer (1990), as - fluid phase and receptor mediated endocytosis.

Coated vesicles were first demonstrated in plant cells from root hairs (Bonnet and Newcomb, 1965). Thermodynamical arguments suggests that vesicle-mediated endocytosis may not be possible at turgor pressure > 1 bar (Grandmann and Robinson, 1989). Cram (1980) stated that endocytosis could not be "the principal means" of transporting major nutrients across the plasmalemma. Recent considerations have pointed to the importance of vesicle size in endocytosis (Raven, 1987; Saxton and

Breidenbach, 1988). When endocytic vesicles are of the size of coated vesicles, much less volume is taken and the rise in turgor is then minimal.

The huge fluctuations and variability in turgor pressure can be encountered in plants where in some instances leaf turgor is reported to drop to 0.7 bar under saline conditions (Neumann *et al.*, 1988) and zero or even negative turgor in leaves in field conditions (Turner, 1974).

There is now substantial evidence for endocytosis in higher plants (reviewed by Robinson and Hillmer, 1990). This phenomenon had been demonstrated with suspension culture cells (Hillmer *et al.*, 1989), with intact roots (Oparka *et al.*, 1988) and with excised tissue (Oparka and Prior, 1988; Hillmer *et al.*, 1990).

## **1.12 *In vitro* fertilisation:**

*In vitro* fertilisation can take place in three ways. Stigma fertilisation, placental fertilisation, and fertilisation of isolated ovule without placenta. These techniques allow the study in detail of the process of fertilisation and embryogenesis.

Using stigma fertilisation success has been achieved with many plant species. In the past few years, successful stigmatic pollination has been achieved in *Trifolium* species (Richards and Rupert 1980) and *Glycine max* (L.) (Tilton and Russell, 1983). However, when stigma and style barriers are likely to hinder *in vitro* fertilisation, pollination of the stub is a successful alternative. In order to further combat barriers to crossability, intraovarian pollination and test-tube fertilisation are reported to be additional promising techniques (Maheshwari and Kanta, 1964). On the other hand, the most widely used technique of *in vitro* fertilisation is placental pollination. The method of direct pollination of ovules has succeeded in many plant species as reviewed in Zenktele (1990).

The success or failure of techniques mentioned above is likely to be influenced by a complex of factors, each of which remain a pre-requisite for successful *in vitro* fertilisation. The correct physiological and morphological state of the pollen, the choice of nutrient medium, sterilisation techniques, and *in vitro* growth conditions may be decisive factors.

### **1.13 Tissue culture techniques:**

The purpose of this part of the study programme was to review the general protocols employed in the field of tissue culture, with regards to the handling of plant material, nutritional requirements, sterilisation techniques and the necessary conditions for shooting and rooting. Special emphasis was put on ovary culture.

Murashige and Skoog (1962) medium is the most frequently used medium in plant tissue culture. A concentration of 1-5% of sucrose is usually used *in vitro* depending on the type and age of growth material. Agar is often used as a gelling agent in most media at a concentration of 0.6-0.8%. The most commonly used disinfectant is sodium hypochlorite (NaClO). Chemical sterilisation can be made more effective by placing the plant material in 70% alcohol for few seconds. The addition of Tween 20 or 80 to the sterilising fluid (0.08-0.12%) lowers the surface tension and allows better surface contact.

### **1.14 Aims of this research project:**

The initial hypothesis on which this research project is based is that, since animal eggs have proved to be extremely valuable experimental tools for genetic manipulation *in vitro* (i.e. nuclear transplants - ingestion of DNA/genes), similar

advances in understanding in plant biology can be achieved by developing the methodology of *in vitro* gamete manipulation and fertilisation.

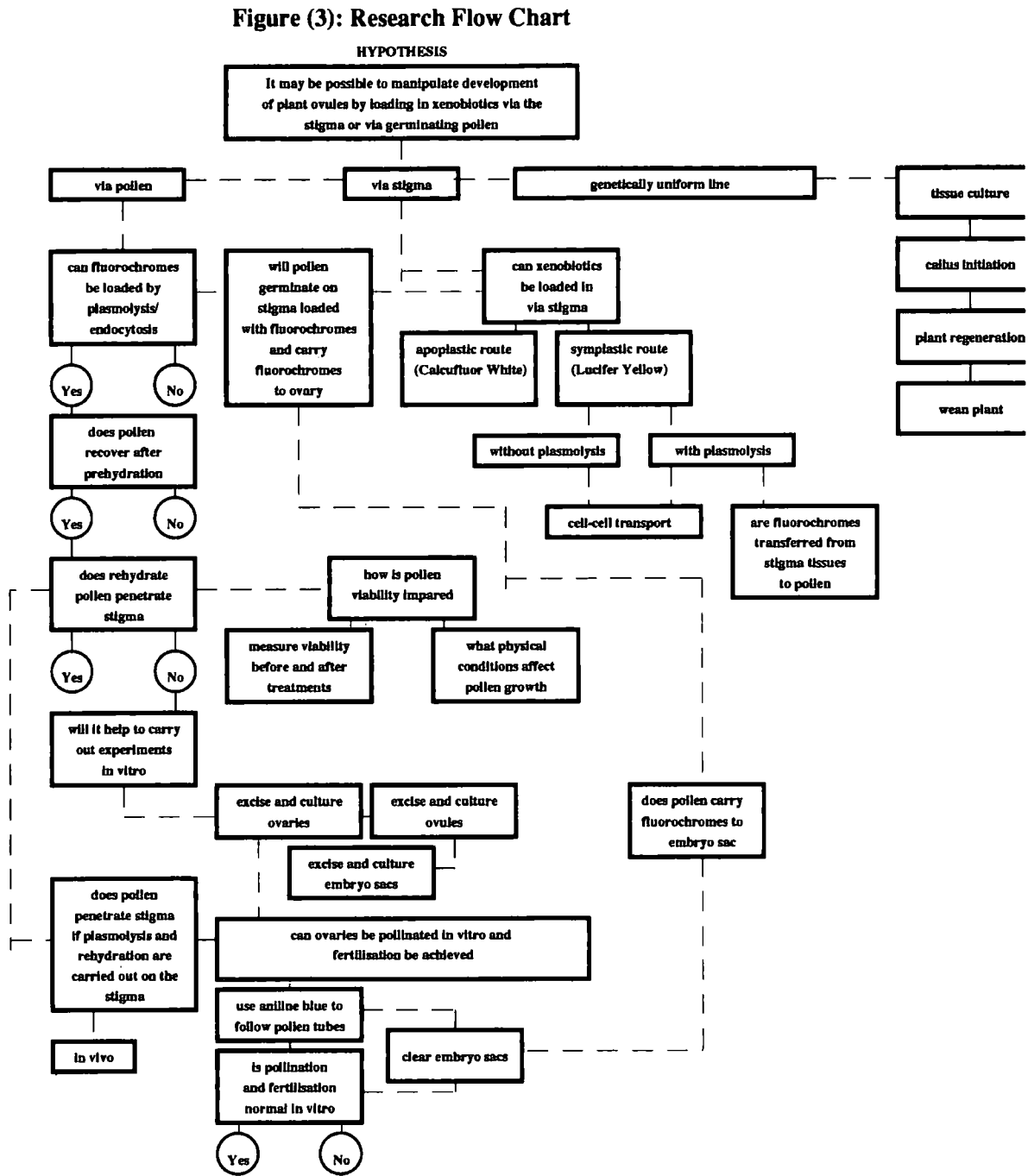
Within a research project of this kind, certain limitations need to be recognised from the outset. Ideally, a study of this type would be conducted on a single plant species. However, logistical difficulties associated with plant growth characteristics and limitations imposed by the physiological and anatomical characteristics of a single plant species meant that more could be achieved by using several plant species, exploiting their individual advantages to advance understanding of the various different phenomena under investigation. For this reason, different plant species were chosen for different aspects of this study, exploiting their individual advantages to achieve the most rapid experimental advances in the time available.

It may be possible to manipulate development of plant ovules similarly by loading in xenobiotics via the stigma or via germinating pollen. The aim of this project was to use *in vitro* germinated pollen from *Impatiens glandulifera* as a means of transferring externally applied, biologically active or inactive molecules to the ES of flowering plants. The study aimed to determine whether pollen germinated in a medium containing fluorescent probe would incorporate this material into membrane bound vesicles when the pollen was subjected to osmotic shock, and to determine whether the germinated pollen could then be transferred to the stigma or into the ovaries of flowers which have been cultured *in vitro*.

After fertilisation, ESs would be examined for traces of the fluorescent probe, signifying successful transfer of the externally applied molecules. If successful, this method would offer a valuable means of experimentally manipulating early embryogenesis and would also be of great practical value in plant breeding. The research project objectives, illustrated in Figure (3), are outlined as follows:

- ① **Assessment of the effect of nutrient elements, temperature and humidity on pollen tube growth *in vitro***
- ② **Assessment of pollen viability**
- ③ **Assessment of pollen developmental state and monitoring of pollen nuclei**
- ④ **Identification of pollen tubes**
- ⑤ **Loading of fluorochromes into pollen by plasmolysis /endocytosis**
- ⑥ **Loading of fluorochromes into plant reproductive cells via the vascular system**
- ⑦ **Manipulation of ovaries/ovules and clearance of ESs**
- ⑧ ***In vitro* ovule culture**
- ⑨ ***In vitro* fertilisation**
- ⑩ **Production of genetically uniform lines**

Figure (3) Research Flow Chart:



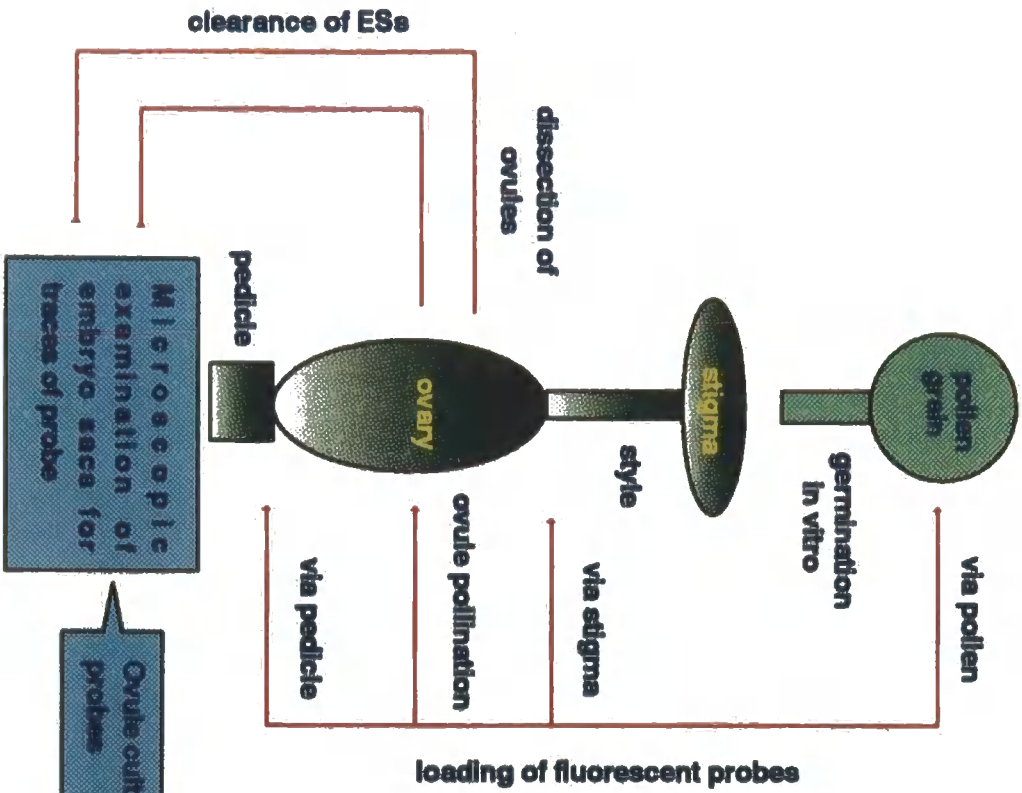
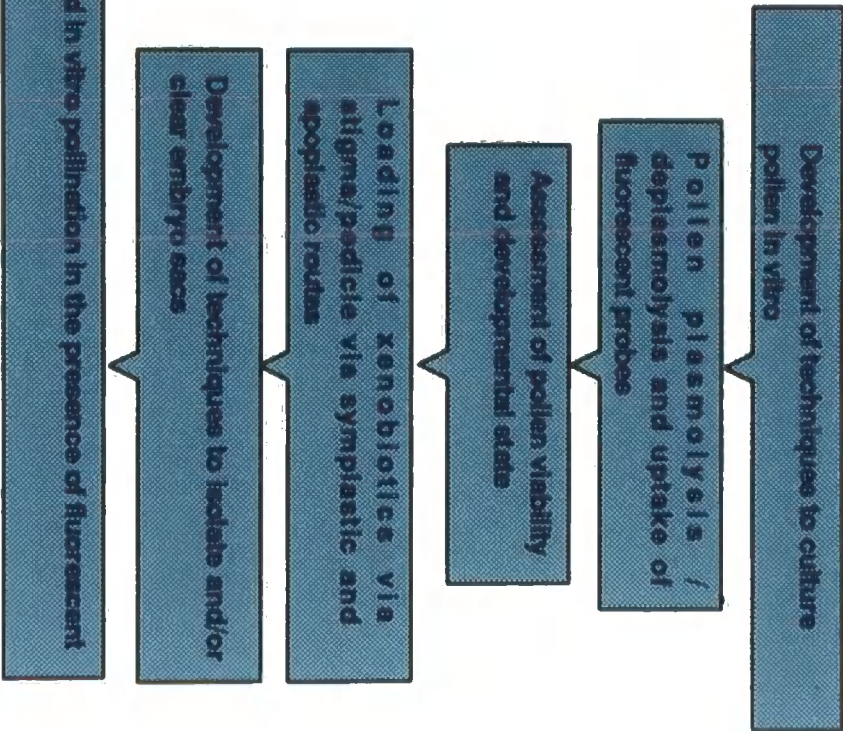


Figure (3) Research Flow Chart

**Hypothesis**

It may be possible to manipulate development of plant ovules by loading in xenobiotics via the stigma or via germinating pollen



## CHAPTER TWO

### MATERIALS AND METHODS

#### 2.1 Biological Material, Chemicals and Culture Media:

##### 2.1.1 Biological Material:

Most experimental work was carried on *Impatiens glandulifera* (*Balsaminaceae*) naturally growing at the Science Site of Durham University during the summer. Additional plant material was made available by growing *I. glandulifera* off-season to provide sufficient material during winter. Seeds collected from previous seasons were sterilised by 70% ethanol for 1 min, 10% sodium hypochlorite for 10 min and washed thoroughly in sterilised distilled water. The seeds were then grown on moistened filter paper in Petri-dishes and incubated in an incubator for two months under 4 °C in order to break seed dormancy, as reported by Mumford (1988). The germinated seeds were then transplanted in compost (Potting and Bedding compost, Agrochemicals, Surrey, UK.) and allowed to grow in the Botanic Gardens of Durham University under 18-22 °C. Daylight illumination was supplemented with sodium lights (400W SONT) for 14h/day.

Other plant material included *Brassica napus* and *Nicotiana tabacum*. The latter species was donated by Kew Gardens and both species were grown in the Botanic Garden of Durham University. Seeds of the former species was provided by Kew Gardens and were germinated on MS culture medium supplemented with 1% (w/v) agar and transplanted to the Botanic Gardens after germination.



## **2.1.2 Chemicals:**

**Supplied by BDH Chemicals Ltd, Poole, England**

Acetic acid

Acridine orange

Aniline blue

Boric acid

Chloral hydrate

Ethanol

Fuchsin basic

Lactic acid

Magnesium sulphate

Phenol

Potassium metabisulphite

Potassium nitrate

Sodium sulphide

Sodium-cacodylate

Sucrose

Xylene

**Supplied by Koch-Light Laboratories Ltd, England**

Calcium nitrate

**Supplied by Difco Laboratories, Detroit Michigan, USA**

Agar

**Supplied by Sigma Chemical Co., St. Louis, USA**

$\alpha$ -Naphthaleneacetic acid

4',6-Diamidino-2-phenylindole

6-Benzylaminopurine

Clove oil

Driselase

Fluorescein diacetate

L-Glutamine

Lucifer Yellow carbohydrazide

Mannitol

Propionic acid

Pyridoxine (Vitamin B<sub>6</sub>)

Thiamine (Vitamin B<sub>1</sub>)

**Supplied by Polysciences Inc., Warrington, PA, USA**

Calcofluor white M2R new

**Supplied by Hopkin & Williams Ltd., Essex, England**

Glycerine

**Supplied by The London Resin Co. Ltd. Hampshire, England**

LR White

**Supplied by Agar Scientific Ltd., Essex, England**

Glutaraldehyde

**Supplied by Hays Chemical Distribution Ltd., County Durham, England**

Sodium hypochlorite

**Supplied by TAAB Laboratories, Reading Berks, England**

Paraformaldehyde

**Supplied by American National Can, Greenwich, C.T.**

Parafilm

**Supplied by Bibby Sterilin Ltd., Stone, Staffs, UK.**

Sterilised plastic Petri-dishes

### 2.1.3 Culture media

#### 2.1.3.1 Murishige and Skoog (MS) basal medium (MS0) and other MS based media (MS1, MS2 and MS3) (supplied by Sigma Chemical Company, St. Louis, USA), used in this study.

Medium	MS0	MS1	MS2	MS3
Component	mg <sup>-1</sup>	mg <sup>-1</sup>	mg <sup>-1</sup>	mg <sup>-1</sup>
Ammonium nitrate	1650.0	1650.0	1650.0	1650.0
Boric acid	6.2	6.2	6.2	6.2
Calcium chloride (anhydrous)	332.2	332.2	332.2	332.2
Cobalt chloride.6H <sub>2</sub> O	0.025	0.025	0.025	0.025
Cupric sulphate.5H <sub>2</sub> O	0.025	0.025	0.025	0.025
Ethylendiamine acidic (disodium salt).2H <sub>2</sub> O	37.26	37.26	37.26	37.26
Ferrous sulphate.7H <sub>2</sub> O	27.8	27.8	27.8	27.8
Magnesium sulphate	180.7	180.7	180.7	180.7
Manganese sulphate	16.9	16.9	16.9	16.9
Molybdic acid (sodium salt).2H <sub>2</sub> O		0.25	0.25	0.25
Potassium iodide	0.83	0.83	0.83	0.83
Potassium nitrate	1900.0	1900.0	1900.0	1900.0
Potassium phosphate monobasic	170.0	170.0	170.0	170.0
Sodium molybdate dihydrate	0.25			
Sodium phosphate monobasic (anhydrous)		148.0	148.0	
Zinc sulphate.7H <sub>2</sub> O	8.6	8.6	8.6	8.6
Adenine hemisulphate		80.0	80.0	
Indole-3-acetic acid (IAA)		2.0		0.3
myo-Inositol		100.0	100.0	100.0
Kinetin		2.0	1.0	1.0
α-Naphthaleneacetic acid (NAA)			0.1	
Sucrose		30000.0	30000.0	30000.0
Thiamine HCl		0.4	0.4	0.4

**2.1.3.2 Nitsch and Nitsch Basal Salt Mixture (2.1 g l<sup>-1</sup>) supplemented by 50g l<sup>-1</sup> sucrose and 9G L<sup>-1</sup> agar(supplied by Sigma Chemical Company, St. Louis, USA), , used in this study.**

Component	mg l <sup>-1</sup>
Ammonium phosphate monobasic	720.0
Boric acid	10.0
Calcium chloride (anhydrous)	166.0
Cupric sulphate.5H <sub>2</sub> O	0.025
Ethylenediaminetetraacetic acid, 2Na.2H <sub>2</sub> O	37.3
Ferrous sulphate.7H <sub>2</sub> O	27.8
Magnesium sulphate	90.37
Manganese sulphate	16.93
Molybdic acid (sodium salt).2H <sub>2</sub> O	0.25
Potassium nitrate	950.0
Potassium phosphate monobasic	68.0
Zinc sulphate.7H <sub>2</sub> O	10.0
Sucrose (30 g l <sup>-1</sup> )	

**2.1.3.3 Gamborg's B-5 basal medium according to Gamborg *et al.* (1968), , used in this study.**

Components	mg l <sup>-1</sup>	
	GM1	GM2
Ammonium sulphate	134.0	134.0
Boric acid	3.0	3.0
Calcium chloride anhydrous*	113.240	113.240
Cobalt chloride hexahydrate	0.0250	0.0250
Cupric sulphate pentahydrate	0.0250	0.0250
Disodium EDTA dihydrate	37.250	37.250
Ferrous sulphate heptahydrate	27.850	27.850
Magnesium sulphate anhydrous**	122.090	122.090
Magnesium sulphate monohydrate	10.0	10.0
Potassium iodide	0.750	0.750
Potassium nitrate	2500.0	2500.0
Sodium molybdate dihydrate	0.250	0.250
Sodium phosphate monobasic anhydrous***	130.50	130.50
Zinc sulphate heptahydrate	2.0	2.0
myo-Inositol		100.0
Nicotinic acid		1.0
Pyridoxine HCl		1.0
Thiamine HCl		10.0
Sucrose (30 g l <sup>-1</sup> )		

\*Original formula contains calcium chloride dihydrate at 150.0 mg l<sup>-1</sup>

\*\*Original formula contains magnesium sulphate heptahydrate at 250.0 mg l<sup>-1</sup>

\*\*\*Original formula contains sodium phosphate monohydrate mg l<sup>-1</sup>

## 2.2 Techniques and Protocols:

### 2.2.1 Measurement of pollen tube length by MicroScale Image Analyser (MSIA)<sup>1</sup>:

- (a) The MicroScale Image Analyser (MSIA) was calibrated to scale using the calibrate function.
- (b) Slides containing germinating pollen were viewed under a Nikon DIAPHOT-TMD Inverted microscope to which a video camera was attached.
- (c) The image from the pollen tubes was grabbed on screen and pollen tubes were measured using the dimension function on the MSIA, which allows for polygonal lines to be drawn along pollen tubes, precisely following their changes in direction.

### 2.2.2 Evaluation of pollen viability by FDA:

- (a) A fresh solution of 5 mg FDA dissolved in 1 ml acetone was prepared.

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#### <sup>1</sup>MSIA FUNCTIONS:

**Calibrate Function:** provides operations that prepare the image for measurement, by setting calibration scales and by manually or automatically picking out the features of interest to be analysed, (e.g. colour thresholding, setting scale, region).

**Dimension Function:** Allows measurement of normal (straight) or poly lines.

**Scan Objects Function:** Causes MSIA to search, detect and measure all discrete objects in the current threshold region of interest.

**Object Stats Function:** Shows the various object parameters for which statistics may be calculated.

(b) Drops from the solution were added to 0.5 M sucrose solution until it became cloudy.

(c) Pollen was immersed in the solution in (b) for 5 min and examined by fluorescence microscopy.

(d) Images were grabbed on screen and fluorescence intensity was measured using the MSIA as described in 2.2.3.

### **2.2.3 Assessment of pollen viability by MicroScale Image Analyser:**

(a) The MicroScale Image Analyser was calibrated using the Calibrate Function (e.g. defining region of interest, colour thresholding)

(b) Image from fluorescent pollen was grabbed on screen and colour intensity was measured using the Scan Function.

(c) Data, showing colour intensity and counts (number of pollen grains), was collected using the Object Stats Function.

### **2.2.4 Preparation of Toluidine blue staining solution:**

(a) A 1% (w/v) solution of Toluidine blue (for 100 ml) was prepared in 0.05% (w/v) citrate buffer (pH 4.0).

(b) The buffer was prepared as follows: 15.4 ml (0.1 M) citric acid + 9.6 ml (0.2 M)  $\text{Na}_2\text{HPO}_4$ .

### **2.2.5 Identification and monitoring of pollen nuclei using DAPI:**

- (a) DAPI was prepared as 1 mg/ml stock solution in phosphate buffer and stored at 4 °C.
- (b) One microlitre of the stock solution was diluted in 1 ml of citrate-phosphate buffer (pH 4.0).
- (c) Pollen was immersed in the solution for 5-10 min and a coverslip applied.
- (d) Observations were made with fluorescence microscopy using a blue excitation filter.

**2.2.6 Preparation of Feulgen staining solution to study pollen developmental state:**

- (a) Anthers were fixed for 24 h in ethanol:acetic acid (3:1).
- (b) After removal of the fixative, they were hydrolysed with 1N HCl for 8 min at 60 °C.
- (c) The HCl was removed and replaced with Schiff's reagent prepared from Fuchsin base (1 g), potassium metabisulphite ( 2 gm), 1N HCl (15 ml) and H<sub>2</sub>O (85 ml) and kept overnight in refrigerator and observed the next day directly in the stain.

**2.2.7 Preparation of CFW stain and detection of cellulose:**

- (a) A 1% (w/v) stock solution of CFW (M2R) was prepared in 0.02 M phosphate buffer (pH 8.0) and stored in the dark at 4 °C.
- (b) Stock solution was diluted to 0.005% prior to use on plant material.



- (c) Plant material was immersed in CFW solution and examined by fluorescence microscopy using an ultra-violet (UV) excitation filter.

### **2.2.8 Technique for staining pollen tubes in stigmas and styles:**

- (a) A 1% w/v aniline blue solution was prepared by dissolving the dye in 1M tri-potassium phosphate and was kept in a refrigerator, in darkness.
- (b) Stigmas, styles and ovaries were first fixed in 70% ethanol.
- (c) Plant material was then hydrolysed for 1 h at 60 °C (using a water bath) in 4% (w/v) sodium hydroxide.
- (d) The specimen was mounted in a drop of decolourised aniline blue for 10-15 min, gently squashed and viewed with the fluorescence microscope using a blue-violet (BV) excitation filter.

### **2.2.9 Preparation of culture media:**

- (a) Approximately 90% of the final required volume of tissue culture distilled water was measured in a container twice the size of the final volume.
- (b) The desired weight of powdered medium was added while stirring the water.

- (c) The desired weights of heat stable substances (e.g. sucrose, agar, vitamins, auxins, cytokinins, etc.) were added. Heating of the solution was carried until clarity of the solution was obtained.
- (d) Additional distilled water was added to bring the medium to the final volume.
- (e) pH was adjusted using NaOH or HCl.
- (f) The medium was then autoclaved at 121 °C for 20 min.
- (g) The medium was allowed to cool prior to use.

#### **2.2.10 Preparation of NAA and BA stock solution:**

- (a) 500 mg of NAA were dissolved in 20 ml of 50% ethanol. 40 µl of the stock solution were added to 1 litre of the basal medium to make 1 mg NAA l<sup>-1</sup>.
- (b) 126 mg of 6-BAP were dissolved in 1 ml 1N HCl. 99 ml of distilled water were added. To make 1 mg l<sup>-1</sup>, 800 µl of the stock solution were added to 1 litre of the basal medium.

#### **2.2.11 Sterilisation of plant material:**

- (a) Sterilisation of plant material was first done by placing the plant material in 70% ethanol for a few seconds.
- (b) Plant material was then placed in a medium consisting of 10% sodium hypochlorite and 1% Tween 80 for 5 min (ovaries) or 10 min (stem cuttings).

(c) Finally, the material was thoroughly washed in sterilised distilled water. Dissections were performed on the sterilised stage of a dissection microscope in a laminar-flow hood.

#### **2.2.12 Clearance of ESs from ovaries using fixing and clearing solutions:**

(a) The fixative was prepared from propionic acid, formaldehyde, ethanol (50%) in the ratio 5:5:90 .

(b) The clearing solution was prepared from lactic acid (85%), chloral hydrate, phenol, clove oil and xylene in the ratio 2:2:2:2:1 by weight.

(c) Plant ovaries were fixed for 24 hr and then immersed in the clearing solution for 24 h at room temperature ( $24\pm 1$  °C).

(d) Ovules were dissected under a stereomicroscope and transferred with a small amount of clearing fluid to slides and examined using a Nikon Optiphot-2 microscope.

#### **2.2.13 Maceration of ESs by enzymic solution:**

(a) The enzyme solution was prepared from 2% driselase, 0.65 M mannitol and 0.25% potassium dextran sulphate.

(b) Ovules were incubated in 1 ml of enzyme solution and placed in a microshaker for 6 h.

- (c) After incubation, plant material was centrifuged for 5 min (1500 rpm) and the pellet was washed 3 times using a washing solution of 0.65 M mannitol and examined under the microscope.

#### **2.2.14 Identification of callose in megasporogenesis:**

- (a) Ovaries were fixed in ethanol-acetic acid (3:1).
- (b) Fixed ovules were hydrolysed in 1 N HCl for 5-10 min at room temperature ( $24\pm 1$  °C), then rinsed in distilled water.
- (c) Callose was then identified in ovule squashes by the callose/aniline-blue method (see Protocol 2.2.8).

#### **2.2.15 Protocol for fixing, dehydrating and embedding of plant material in LR White:**

- (a) Plant material was dehydrated in 25% , 50% , 75% , 90% and 100% ethanol for 1 h each. Dehydration was carried at RT on a 45 ° rotating platform at 2 r.p.m. and the solutions were changed every 30 min.
- (b) An equal volume of LR White acrylic resin (medium grade) was added to the dehydrated samples under 100% ethanol. The solutions were mixed thoroughly and incubated overnight, with gentle agitation at room temperature.
- (c) The 50:50 resin:ethanol mixture was then replaced by resin alone and incubated at RT for a further 4 h. The resin was then replaced

twice daily until the tissues were judged to be fully infiltrated.  
Resin blocks were polymerised overnight at 65 °C.

(d) Sections (4 µm thick) were made using MT2-B Ultra Microtome  
(Kvan Sorvall Inc. Norwalk Conn. USA).

**2.2.16 Examination of plant material by fluorescence microscopy:**

<b>Test</b>	<b>Probe</b>	<b>Excitation method</b>	<b>Dichroic mirror</b>	<b>Barrier filter</b>
<i>Pollen viability</i>	<b>Fluorescein diacetate</b>	<b>Blue filter EX450-490</b>	<b>DM510</b>	<b>BA520</b>
<i>Identification of callose in pollen tubes and in ovules</i>	<b>Aniline blue</b>	<b>Blue-violet filter EX400-440</b>	<b>DM455</b>	<b>BA470</b>
<i>Detection of cellulose</i>	<b>Calcofluor White</b>	<b>Ultra-violet filter EX330-380</b>	<b>DM400</b>	<b>BA420</b>
<i>Pollen viability</i>	<b>DAPI</b>	<b>Ultra-violet filter EX330-380</b>	<b>DM400</b>	<b>BA420</b>
<i>Identification and monitoring of pollen nuclei</i>	<b>Acridine orange</b>	<b>Blue filter EX450-490</b>		
<i>Uptake of probe by pollen and vascular tissue</i>	<b>LY-CH Fluorescein</b>	<b>Blue filter EX450-490</b>	<b>DM510</b>	<b>BA520</b>

### **2.2.17 Computer data handling programmes:**

All measurements are carried by MicroScale TM/TC Image Analyser (MSIA). The programme is a PC-based image analysis system with Digithurst's proprietary MicroEye TM and TC transputer based framestore image processing cards. The system is calibrated by setting calibration scales. The threshold operation in the system allows the operator to calibrate the range of intensities, or colours to be measured (MicroScale Image Analysis System User Guide, Digithurst Ltd, 1990). The MicroEye TM/TC card holds the captured image as a 720 (horizontal) by 512 (vertical) matrix of pixels. Each pixel in monochrome mode takes a value from 0 to 255 (bit range) representing its brightness level (0 = black, 255 = white).

Data statistical analysis was carried using the PC-based Minitab Statistical Analysis Programme. Statistical results included the determination of standard deviation, standard error, and analysis of variance.

### **2.2.18 Photography:**

Two Nikon microscopes were used in photomicrography, the DIAPHOT-TMD with a 35mm camera (Nikon FE) on the front of the microscope and the OPTIPHOT-2 with photomicrographic attachment (FX-35) on the top. Fujicolor 400 films (ISO 400/27°) were used, exposed and processed according to the manufacturer recommendations.

## 2.3 Manipulation of pollen *in vitro*

### 2.3.1 Assessment of the role of nutrient elements on pollen growth *in vitro*:

**OBJECTIVE:** Fresh pollen was germinated in a range of sucrose concentrations from 5-20% (w/v) in an attempt to study the nutritional and/or osmotic role of sucrose in pollen tube growth. Pollen growth was measured after incubation in a nutrient medium consisting of boric acid only (0.005-0.025% (w/v)). The optimum sucrose concentration obtained, as described earlier, was combined with different concentrations of boric acid (0.005-0.025% (w/v)). The objective, in this regard, was to assess the combined role of both nutrient elements. Having derived the optimum concentration of sucrose and boric acid, the nutrient medium was supplemented with calcium nitrate, magnesium sulphate and potassium nitrate.

In order to avoid discrepancies brought about by genetic and physiological differences between plants, and between flowers of the same plant as far as possible, pollen from a single flower was used. Three culture techniques were first compared by culturing pollen in a medium according to Brewbaker and Kwack (1963) for 15 min using hanging-drop, sitting-drop and cellophane sheets placed on filter paper moistened with medium.

The technique adopted in this experiment was a sitting-drop technique in which fresh pollen were immersed for 1 hr in nutrient media composed of sucrose (5, 10, 20% (w/v)) and boric acid (0.005% (w/v), 0.01% (w/v), 0.02% (w/v)) solutions. The effect of the optimum concentration of sucrose on pollen growth was further evaluated in combination with different concentrations of boric acid. The optimum combination of sucrose and boric acid derived from the previous experiment was further supplemented with 100 ppm calcium nitrate, 100 ppm potassium nitrate and 100 ppm magnesium sulphate.



In each treatment, 20 randomly selected pollen tubes were measured using the MSIA (Protocol 2.3.1) one hour after incubation of pollen at room temperature ( $24\pm 1$  °C). To assess pollen tube growth rate in liquid and solidified pollen culture media (PCM), fresh pollen was cultured in both media and tube length was measured from 50 randomly selected pollen 15 min, 30 min 1 h and 2 h after incubation at room temperature.

### **2.3.2 Effect of temperature and humidity on pollen growth *in vitro*:**

In order to assess the effect of temperature on pollen growth in terms of tube length and germination percentage, pollen from a freshly opened floral bud were incubated for 6 h at 4 °C, 8 °C and room temperature (25 °C) on cellophane sheets placed on filter paper moistened with culture medium . Measurement of pollen tube length was carried out using MSIA on 50 randomly selected pollen tubes and the mean germination percentage was measured from five replicates. At a later stage, the cellophane sheet technique was replaced by a technique in which pollen were incubated in culture medium supplemented with 1% (w/v) agar and pollen tube length and germination percentage were measured at intervals of 2-4 h at room temperature. The reasons for change in culture technique were two folds. Firstly, repeated removal of cellophane sheets for measurements led to drying out of pollen, which directly affected its growth. Secondly, solidified media allowed pollen culture for longer periods, as well as making the application of staining tests on germinated pollen more convenient.

The effect of relative humidity on pollen growth was studied using three levels of relative humidity simulated by saturated solutions (Winston and Bates, 1960). Saturated solutions of glucose, NaCl and KNO<sub>3</sub> were made to give relative humidity of 55.0, 75.0 and 92.5 respectively. The solutions were prepared by dissolving enough solid to saturate at boiling and allowing it to cool. More solid was added and the

solution was allowed to stand for 3-4 days to insure saturation. Room temperature was kept constant and maintained using a water bath. These particular solutions were selected because they were reported to be consistent over a wide range of temperatures. Pollen were dusted on cellophane sheets which were placed on filter paper moistened with culture medium. These were then placed in Petri dishes which were allowed to float on a closed vessel consisting of the saturated solution.

To assess the effect of relative humidity on pollen growth, fresh batches of pollen were incubated for 24 h at three RH levels (55.0, 75.0 and 92.5). They were cultured in solidified PCM for 90 min and pollen tubes were measured by MSIA.

### **2.3.3 Assessment of pollen viability at different temperature and humidity conditions:**

**OBJECTIVE:** Pollen viability testing was carried out using Fluorescein Diacetate, CFW New, with and without plasmolysis/deplasmolysis. The response of pollen to these treatments was carried under different temperature and humidity conditions.

A stock solution of CFW was prepared as 1% w/v in 0.02 M phosphate buffer (pH 8.0) and stored in the dark at 5 °C. Pollen was immersed for 5 min in two concentrations of CFW (0.1 and 1% w/v). Pollen was incubated for 24 h under 4 °C, 8 °C, room temperature and 60 °C and then immersed in 0.1% (w/v) CFW for 5 min and fluorescence intensity was measured from 100 pollen grains, randomly selected.

The same test was carried out using FDA instead of CFW. FDA was prepared as 5 mg/ml in acetone. Fluorescence intensity was measured after incubation of fresh pollen as mentioned above. In both cases, measurement of fluorescence intensity was carried out on grabbed images, using a Nikon DIAPHOT-TMD Inverted Microscope with fluorescence attachment (Nippon Kogaku K.K., Tokyo, Japan) under blue excitation for FDA and ultraviolet excitation for CFW.

Three species, *I. glandulifera*, *N. tabacum* and *B. napus* were tested using the FCR test on fresh pollen by measuring fluorescence intensity using MSIA. Pollen area and diameter were also measured. To assess the effect of temperature on pollen viability, pollen from *I. glandulifera* were preincubated at 4 °C, 8 °C, RT and °60 C. Pollen was then treated with CFW and FDA for 5 min as mentioned above.

#### **2.3.4 Assessment of pollen state and monitoring of pollen nuclei:**

**OBJECTIVE:** To study pollen developmental state at different growth stages using the Feulgen staining and DNA-specific probes.

To assess pollen state in *I. glandulifera*, floral buds (0.4 cm - 1.2 cm long) were collected to represent different growth stages from the initial stage up to the stage where the anther splits open. Buds were treated by Feulgen staining as described in Protocol (2.3.6). They were then crushed in a drop of distilled water and examined under the Inverted Microscope. Pollen state was further assessed using acridine orange, DAPI and aniline blue as described in the techniques and protocols section.

In order to monitor the behaviour of pollen nuclei *in vitro*, pollen was cultured in the basal medium previously described with the addition of 0.01 mg ml<sup>-1</sup> of the DNA-specific probe DAPI. Pollen nuclei were examined microscopically using a Nikon Optiphot-2 Fluorescence Microscope under an ultra-violet filter (EX330-380, DM400, BA420) and photographs were taken as described earlier (2.2.19).

#### **2.3.5 Loading of fluorochromes into pollen by plasmolysis / endocytosis:**

**OBJECTIVE:** Pollen was plasmolysed using mannitol to obtain endocytic vesicles. Pollen was then allowed to grow *in vitro* in the culture medium described earlier. The effect of plasmolysis was studied by germination of plasmolysed pollen and measurement of pollen tube length. The feasibility of uptake of fluorescent probes by pollen was assessed by plasmolysing pollen in the presence of fluorescent probe (LY-CH) and deplasmolysing it. Experiments were conducted to trace the probes into the pollen. Three batches of fresh

pollen were compared; a control batch, plasmolysed and deplasmolysed in mannitol, and plasmolysed and deplasmolysed in the presence of fluorescent probe. These batches were fixed, dehydrated and embedded in LR White, sectioned and examined microscopically. As fluorochromes (LY-CH and FDA) were added to the pollen culture medium described earlier, it is therefore necessary to conduct experiments in an attempt to assess its likely effect on pollen tube growth *in vitro*.

To assess the effect of plasmolysis on pollen growth *in vitro*, fresh pollen from *I. glandulifera* were dusted on cellophane sheets placed on filter paper moistened with 700 mM unbuffered mannitol. Plasmolysis was conducted for 1 h. Pollen were then transferred to a solution containing 300 mM mannitol for 30 min in order to deplasmolyse the pollen. Pollen was then allowed to grow for 45 min on culture medium supplemented with 1% (w/v) agar. A control treatment was made by culturing pollen without plasmolysis.

Pollen was plasmolysed with 700 mM unbuffered mannitol in the presence of LY-CH (5 mg ml<sup>-1</sup>) for 1 h and allowed to deplasmolyse for 30 min in 300 mM mannitol. Another batch of pollen was treated with 1 mg ml<sup>-1</sup> fluorescein solution (pH 7.7). Both batches were examined microscopically as shown in Protocol (2.2.16) prior to culturing on medium supplemented with 1% (w/v) agar. Tube length was measured 45 min after incubation at room temperature. The effect of LY-CH (5 mg ml<sup>-1</sup>) and fluorescein (1 mg ml<sup>-1</sup>) on pollen growth *in vitro* was assessed by measuring pollen growth on culture media containing the probes.

In another batch treated as mentioned above, pollen were thoroughly washed by a fixative solution consisting of 3% glutaraldehyde and 2% paraformaldehyde in 50 mM sodium-cacodylate buffer (pH 8.4) and fixed in the same solution for 5 h. It was then dehydrated through a graded ethanol series (25% , 50% , 75% and 100% ) at intervals of 1 h. Finally, pollen were embedded in London Resin (LR White; Agar scientific, Stanstead, Essex, UK) and serial sections of 1 µm were cut using MT2-B Ultramicrotome, dried and mounted in fluoromount (BDH, Poole, Dorset, UK) and examined under a Nikon Optiphot-2 Fluorescent Microscope under a blue filter

(dichromic mirror 510, excitation filter 450-490 and barrier filter 520) . A control batch of fresh pollen was plasmolysed, deplasmolysed and fixed similarly.

### **2.3.6 Plasmolysis/deplasmolysis of pollen, *in vitro* pollination and identification of pollen tubes:**

**OBJECTIVE:** In an attempt to detect whether *in vitro* pollination using the above mentioned techniques was effective, and to show whether plasmolysed/deplasmolysed pollen could reach ovules, after pollination with control and plasmolysed/deplasmolysed pollen, ovules were dissected and stained with a mixed stain of aniline blue and the fluorescent brightener 'CFW New', after pollination with control and plasmolysed/deplasmolysed pollen.

Pollen tubes were identified *in vitro* and *in vivo* using the technique mentioned in Protocol (2.2.8). The objective at this stage was to devise an effective pollen tube identification technique to be used. However, the consideration of callose plugs as a sensitive indicator of pollen tube growth rate by Snow and Spira (1991) made it worth testing applicability of their findings. Pollen was plasmolysed with 700 mM unbuffered mannitol for 1 h and allowed to deplasmolyse for 30 min in 300 mM mannitol. *In vitro* pollination was conducted with pollen immersed in PCM on stigma-cut ovaries. *In vitro* identification was carried on pollen germinating for 24 h in solidified PCM. Distance up to first and second callose plugs was measured in *I. glandulifera* and *N. tabacum*. using MSIA.

### **2.4 Loading of fluorochromes into plant cells via the vascular system:**

**OBJECTIVE:** To avoid the potentially damaging plasmolysis/deplasmolysis procedure which is necessary for loading fluorochromes into cell by endocytosis, fluorochromes were loaded into plant cell via the vascular system .

Several fluorescent compounds have been used as probes for studying plant transport processes. This study considers the potential mechanisms of uptake of LY-CH, CFW and fluorescein.

A preliminary study, was carried out by embedding plant ovaries and pedicels in LR white as indicated in protocol (2.2.15) and staining sections with toluidine blue in order to study tissue and cell structure.

Petals and stamens were removed from two batches of pistils from *N. tabacum*, *I. glandulifera* and *B. napus* and pedicels were trimmed to about 0.5 cm. Pedicels were then immersed into 1 mg ml<sup>-1</sup> LY-CH for 2 h. In the first batch, pedicels were embedded in LR White as previously described and transverse sections were cut using an ultra-microtome. In the second batch, ovules were dissected under the stereomicroscope. Pedicel sections and ovules were viewed under a Nikon Optiphot-2 Fluorescent Microscope using blue excitation filter (dichromic mirror 510 nm, excitation filter 450-490 nm, and barrier filter 520 nm).

The loading of CFW and fluorescein into plant cells via the pedicel was studied by incubating *Brassica napus* pedicels in 0.1% (w/v) CFW and 5 mg ml<sup>-1</sup> uranin (disodium fluorescein) for 2 h. Transverse sections of pedicels and dissected ovaries were prepared as previously described. Fluorescence microscopic examination was carried out using blue and UV (EX330-380, DM400, BA420) excitation filters for CFW and uranin respectively.

Fluorescein (1 mg ml<sup>-1</sup>) was loaded through the pedicel of *I. glandulifera* by immersing pedicels for 1h and uptake of the probe was followed up to the anther from which pollens were collected and examined by fluorescence microscope before and after culture in liquid PCM.

## **2.5 Manipulation of ovaries/ovules and clearance of ESs:**

**OBJECTIVE:** To develop techniques to clear and/or isolate ESs in an attempt to facilitate examining them microscopically for traces of fluorescent probes loaded via germinating pollen or via the vascular system. These included enzymic maceration, tissue clearing solutions, and fixation, embedding and serial sectioning.

Many techniques has been developed for the study of significant features in the embryology of angiosperms, specifically ovule development, megasporogenesis and megagametogenesis. Each technique, however, reveals specific information and serves certain objectives, which could not be accomplished using other techniques. Nevertheless, some techniques have been devised for particular species. In this study, two techniques were employed on fresh and fixed material from *I. glandulifera* and *N. tabacum* in an attempt to derive a convenient method. These include the clearing technique and the enzymic maceration technique.

### **2.5.1 Clearance of ESs by fixing and clearing solutions:**

The method adopted for fixing and clearing of ESs from *I. glandulifera* and *N. tabacum* ovules is described in Protocol 2.2.12. Pistils from both species were collected from floral buds at the time of anther opening and 1 week after pollinating *in vivo*. Dissected ovules fixed for 24 h were immersed in the clearing solution for 24 h and examined microscopically by differential interference contrast (DIC) microscopy using a Nikon Optiphot-2 Microscope.

### **2.5.2 Isolation of ESs by enzymic maceration:**

Ovules from *I. glandulifera* were excised and macerated enzymically using 2% (w/v) driselase solution (Protocol 2.2.13) for 2-3 h at 24-26 °C. When isolation had occurred, the suspension was centrifuged at 1,500 rpm for 5 min. The supernatant was discarded and the precipitate washed three times by resuspension and recentrifugation with 0.1 M sucrose solution. The isolated embryo sacs were examined for viability using the FDA test.

## 2.6 *In vitro* ovule culture:

**OBJECTIVE:** To develop a suitable medium for ovule culture *in vitro* for further experimental work that requires *in vitro* pollination of ovules and study of subsequent development of embryo sacs.

A preliminary study was carried in an attempt to develop a convenient medium for *in vitro* ovule culture. *In vivo* pollinated ovaries of *N. tabacum* were collected 2 days after anther opening (DAA). Fifteen different media were assessed (Table 12) for ovule development *in vitro*. Ovaries were thoroughly washed in sterile distilled water, surface-sterilised in 70% ethanol for 30 sec and in 1% sodium hypochlorite for 5 min and were cultured in these media in sterilised plastic Petri-dishes and cultured for longer periods to assess their ability to germinate *in vitro*.



**Table (12): Culture media for *N. tabacum* ovule culture *in vitro*:**

Culture Medium	Code
MS (4.703g l <sup>-1</sup> ) <sup>1</sup> +agar (9 g l <sup>-1</sup> )	MS0
MS0 +NAA (0.1 mg l <sup>-1</sup> )	MS1
MS0 +6-BAP (1.0 mg l <sup>-1</sup> )	MS2
MS0 +NAA (0.1 mg l <sup>-1</sup> ) +6-BAP (1.0 mg l <sup>-1</sup> )	MS3
MS0+sucrose (120 g l <sup>-1</sup> ) <sup>1</sup>	MS4
MS0+sucrose (50 g l <sup>-1</sup> ) <sup>2</sup>	MS5
MS4+NAA (0.1 mg l <sup>-1</sup> )	MS6
MS4 +6-BAP (1.0 mg l <sup>-1</sup> )	MS7
MS4 +glutamine (400 mg l <sup>-1</sup> )	MS8
MS6 +6-BAP (1.0 mg l <sup>-1</sup> )	MS9
MS9 +glutamine (400 mg l <sup>-1</sup> ) <sup>3</sup>	MS10
MS9 +Vit1 (0.1 mg l <sup>-1</sup> ) +Vit6 (0.1 mg l <sup>-1</sup> )	MS11
MS11 +NAA (0.1 mg l <sup>-1</sup> ) +6-BAP (1.0 mg l <sup>-1</sup> )	MS12
Nitsch & Nitsch <sup>4</sup> +sucrose (50 g l <sup>-1</sup> ) +agar (9 g l <sup>-1</sup> )	N&N
Gamborg's B-5 basal salt mixture +sucrose (50 g l <sup>-1</sup> ) +agar (9 g l <sup>-1</sup> )	GM1
Gamborg's B-5 basal medium with minimal organics +sucrose (50 g l <sup>-1</sup> ) +agar (9 g l <sup>-1</sup> )	GM1

As shown in Table (12), most of the culture media were based on Murashige and Skoog (1962) medium as full strength. Two levels of sucrose used are 120 gl<sup>-1</sup> (according to Monnier, 1976) and 50 gl<sup>-1</sup>(according to Nitsch and Nitsch, 1969). In some media, however, glutamine and vitamins (according to Monnier and Lagriffol, 1985 and Litz, 1985) were added. The culture media tested also included the Nitsch and Nitsch (1969) medium supplemented with 50 gl<sup>-1</sup> sucrose and 9 g l<sup>-1</sup> agar and Gamborg's B-5 basal salt mixture supplemented with 50 gl<sup>-1</sup> sucrose and 9 g l<sup>-1</sup> agar

<sup>1</sup>Murashige and Skoog (1962)

<sup>1</sup>Monnier (1976)

<sup>2</sup>Nitsh and Nitsch (1969)

<sup>3</sup>Monnier and Lagriffol (1985), Litz (1985)

<sup>4</sup>Nitsch and Nitsch (1969)

(GM1) and Gamborg's B-5 basal medium with minimal organics supplemented with 50 g l<sup>-1</sup> sucrose and 9 g l<sup>-1</sup> agar (GM2) according to Gamborg *et al.* (1968)

## 2.7 *In vitro* fertilisation:

**OBJECTIVE:** To develop an effective method for *in vitro* fertilisation of ovules and/or ovaries cultured *in vitro*, in the presence of and without fluorescent probes.

The techniques of stigmatic pollination and direct pollination of ovules cultured *in vitro* were adopted in this study. The latter technique would bypass all the prefertilisation barriers (Zenktler, 1992). However, while stigmatic pollination does not bypass such barriers, it provides a good system for analysing the role of various media- and environment-related factor on self-incompatibility systems (Douglas and Connolly, 1989). Both techniques are equally appropriate for analysing postzygotic barriers (Zenktler, 1990).

Whole ovaries of *I. glandulifera* were collected at the time of anther opening, sterilised according to protocol (2.2.11) and cultured in MS-based media (2.1.3.1). Anthers were collected before opening, surface-sterilised in 70% ethanol for 30 sec and in 1% sodium hypochlorite for 5 min and allowed to open in sterile plastic Petri-dish. Pollen grains were dusted on stigmas. The ovules were dissected 48 h later and examined for pollen tube penetration using protocol (2.2.14).

The previous pollination technique was also applied to *B. napus* pistils cultured *in vitro*. Pollination was carried out after cutting the stigma and placing the pollen grains (which were plasmolysed and deplasmolysed in the presence of LY-CH) on the style.

Ovaries from *N. tabacum* were collected preanther opening, sterilised as mentioned earlier and cultured in Nitsch and Nitsch (1969) medium. Anthers were collected before opening, washed thoroughly with sterilised water, rinsed in 70% ethanol for 30 sec and in 1% sodium hypochlorite for 5 min. The anthers were then

allowed to open in sterile plastic Petri-dishes. Pollen was prehydrated in a humid environment for 1 h. All sterilisation and pre-conditioning of pollen was carried out the same day as pollen were used for pollinating. A batch of *N. tabacum* ovaries was cultured without pollinating as a control treatment.

## 2.8 Production of genetically uniform line:

**OBJECTIVE:** The main objective at this stage was to develop genetically uniform experimental material by culturing plant tissues *in vitro*. These would be allowed to regenerate into shoots and/or induce callus that could further be subcultured and stimulated for shoot regeneration. Special emphasis would be put on ovule culture as the above mentioned set of *in vitro* pollination experiments are basically carried out on ovules cultured *in vitro*.

Experiments were conducted, in an attempt to, micropropagate *I. glandulifera*, *B. napus* and *N. tabacum*, to produce genetically identical plants. The technique used in these experiments was the single node culture technique in which a bud, together with a piece of stem was isolated.

To reduce the chances of infection, closed buds were isolated. Additional sterilisation measures were made by chemical sterilisation. The plant material was first thoroughly washed in sterilised distilled water, then placed in 70% ethanol for 30 sec and immersed in 10% sodium hypochlorite for 10 min. The plant material was stirred during the utilisation of the bleach to ensure adequate effect. Finally, the plant material was thoroughly washed in sterilised distilled water. The sterilisation procedure was conducted in a sterilised laminar flow chamber.

The culture media [protocol (2.1.3.1) for *I. glandulifera* and protocol (2.1.3.2) for *B. napus* and *N. tabacum*] were supplemented by 0.6% (w/v) agar and prepared according to protocol (2.2.9) and poured in sterile containers. Finally, the plant material was cultured in the media and incubated in a growth room (2x4x4m), 24 h illuminated by 18 (100 W Polylux 300) fluorescent tubes, at room temperature (24±1 °C).

## CHAPTER THREE

### RESULTS

#### 3.1 Manipulation of pollen *in vitro*

##### 3.1.1 Assessment the role of nutrient elements on pollen growth *in vitro*:

The three culture techniques were compared statistically as shown below in Table (1) which reveals a non-significant difference ( $P < 0.05$ ) between culturing pollen in sitting-drop, hanging-drop, or on cellophane sheets placed on culture medium. Pollen tubes lengths obtained with the three techniques are 117.46 ( $\pm 54.94$ ), 115.74 ( $\pm 41.97$ ) and 110.90 ( $\pm 40.75$ ) respectively.

Pollen tube length ( $\mu\text{m}$ ) obtained after incubation of *I. glandulifera* pollen in 5-20% (w/v) sucrose solution for 1 h is shown in Figure (4.1). Statistical results (Table 2) revealed a highly significant difference, with longer tubes obtained with 5% (w/v) sucrose (258.81  $\mu\text{m}$ ) and a decreasing trend with the increase in sucrose concentration from 10-20% (w/v) (203.41 and 23.04  $\mu\text{m}$  respectively).

Boric acid, on the other hand, as shown in Figure(4.2) and Table (3) gave longer pollen tubes (168.97  $\mu\text{m}$ ) at 0.02% (w/v) concentration compared to 0.005% (w/v) and 0.01% (w/v). However, pollen tubes were shorter than those obtained with sucrose only. However, when both sucrose (5% (w/v)) and boric acid (0.005-0.02% (w/v)) were combined, as shown in Figure (5.1) and Table (4), pollen tubes up to 552  $\mu\text{m}$  long were obtained; longer than were obtained using the previous media in which both nutrients were used separately.

The addition of 100 ppm of Mg, Ca, and K (Table 5 & Figure 5.2), however, showed significant variation in the role played by each of these nutrient elements,

particularly with potassium (463.20  $\mu\text{m}$ ). According to these results, the best medium for culture of *I. glandulifera* pollen which provided longer pollen tubes was 5% (w/v) sucrose, 0.01% (w/v) boric acid supplemented by 100 ppm calcium nitrate, 100 ppm potassium nitrate and 100 ppm magnesium sulphate. This medium was used throughout succeeding experiments and will be referred to as the pollen culture medium (PCM).

As shown in Table (6) and Figure (6) pollen tube growth rate in liquid PCM reached a maximum rate after 30 min ( $12.56 \mu\text{m min}^{-1}$ ), while a maximum growth rate in solidified PCM was obtained after 15 min (Table 7). However, growth rate decreased on both media 1 h after incubation until it dropped to 7.49 and 6.45  $\mu\text{m min}^{-1}$  after 2 h in solidified and liquid PCM, respectively (Figure 6). In both cases, germination started in less than 5 min.

**Table (1) Analysis of variance of *Impatiens glandulifera* pollen tube length ( $\mu\text{m}$ ) after incubation in growth medium for 15 min using sitting-drop, hanging-drop and cellophane sheets culture techniques.**

Source	DF	SS	MS	F
Factor	2	579	289	0.13
Error	72	154565	2147	
Total	74	155144		

**Table (2) Analysis of variance of the effect of sucrose concentration (5%, 10%, 20% w/v) on pollen tube length ( $\mu\text{m}$ ) of *I. glandulifera* 1 h after incubation at room temperature.**

Source	DF	SS	MS	F
Factor	2	606798	303399	215.57
Error	57	80224	1407	
Total	59	687022		

Level	Mean	StDev
5% (w/v)	258.51	52.43
10% (w/v)	203.41	37.90
20% (w/v)	23.04	6.07

**Table (3) Analysis of variance of the effect of boric acid (0.005, 0.01%, 0.02% w/v) on pollen tube length ( $\mu\text{m}$ ) of *I. glandulifera* 1 h after incubation at room temperature.**

Source	DF	SS	MS	F
Factor	2	133564	66782	59.46
Error	57	64017	1123	
Total	59	197582		

Level	Mean	StDev
0.005% (w/v)	76.41	20.68
0.01% (w/v)	62.75	36.00
0.02% (w/v)	168.97	40.57

**Table (4) Analysis of variance of the effect of sucrose concentration (5% (w/v)) and boric acid (0.005-0.02% (w/v)) on pollen tube length ( $\mu\text{m}$ ) of *I. glandulifera* 1 h after incubation under room temperature.**

Source	DF	SS	MS	F
Factor	2	718496	359248	20.15
Error	57	1016278	17829	
Total	59	1734774		

Level	Mean	StDev
0.005% (w/v)	284.0	126.3
0.01% (w/v)	552.0	124.4
0.02% (w/v)	416.1	148.5

**Table (5) Analysis of variance of the effect of sucrose(5% (w/v)), boric acid (100 ppm) and Mg, Ca and K (100 ppm) on pollen tube length ( $\mu\text{m}$ ) of *I. glandulifera* 1 h after incubation at room temperature.**

Source	DF	SS	MS	F
Factor	4	623706	20902	37.87
Error	95	417282	5491	
Total	99	1040988		

Level	Mean	StDev
Mg	304.92	96.69
Ca	275.51	62.40
K	463.20	83.46
Mg+Ca+K	712.12	64.98
Control	227.85	41.89

**Table (6) Pollen tube growth rate ( $\mu\text{m}/\text{min}$ ) of *I. glandulifera* after incubation in liquid PCM for 15 min, 30 min, 1 h and 2 h at room temperature.**

Level	Mean	StDev	$\mu\text{m}/\text{min}$
15 min	164.2	41.6	10.94
30 min	376.8	121.4	12.56
1 h	760.3	194.8	12.76
2 h	774.9	160.5	6.45



Figure (4.1): The effect of sucrose on pollen tube growth of *L. glandulifera* 1 h after incubation in liquid PCM.

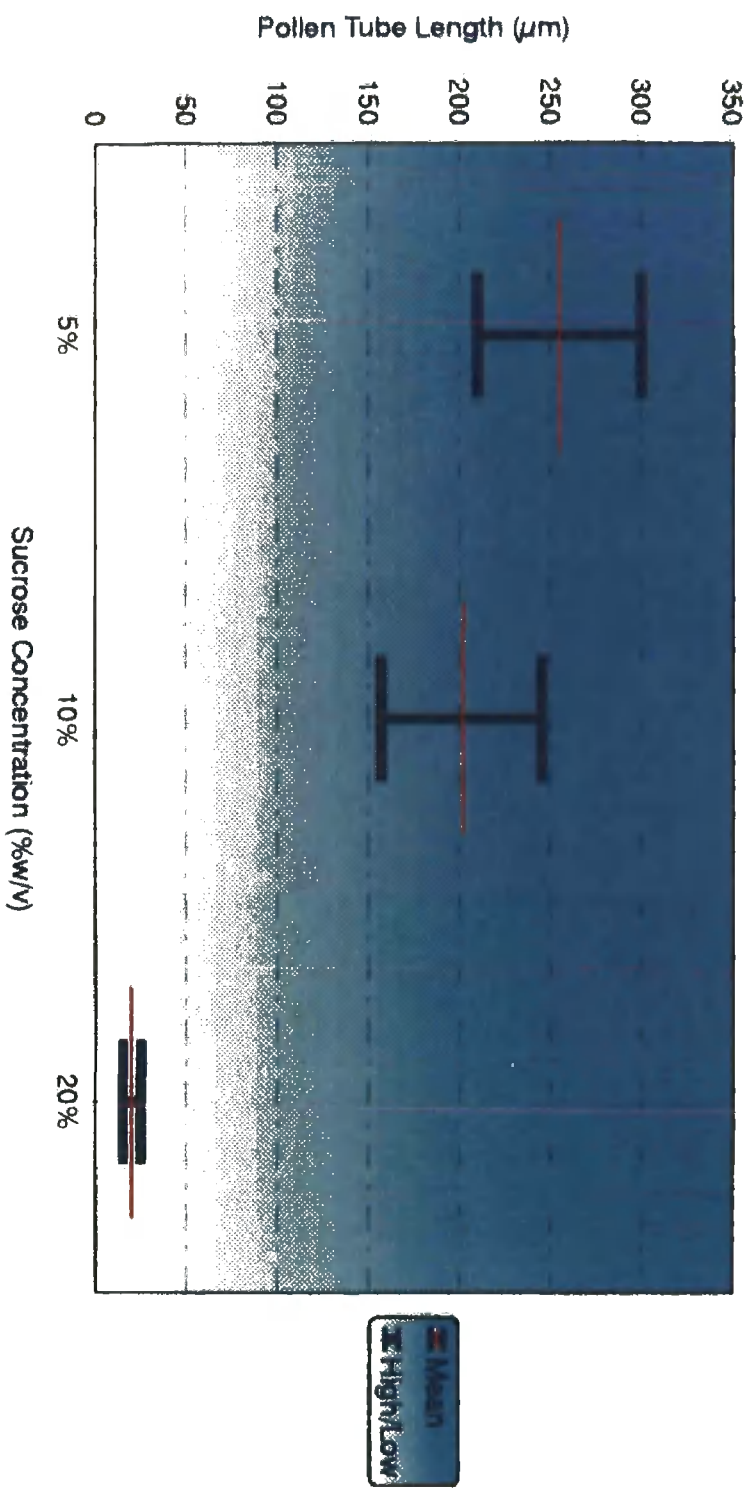


Figure (4.2): The effect of boric acid concentration on pollen tube length of *I. glandulifera* 1 h after incubation in liquid PCM.

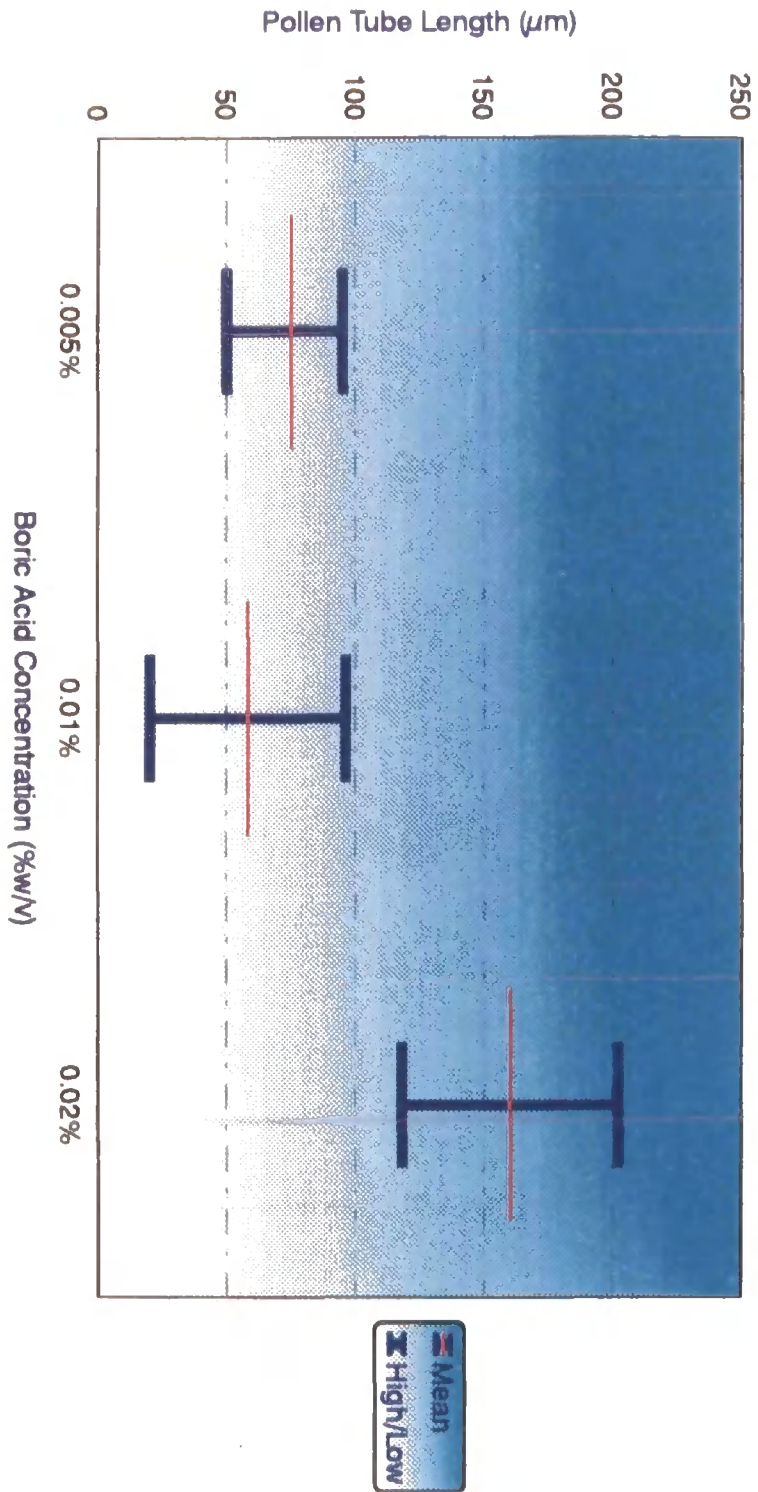


Figure (5.1): The effect of 5% sucrose supplemented with 0.005%, 0.01% and 0.02% boric acid on pollen tube growth of *I. glandulifera* 1 h after incubation in liquid PCM.

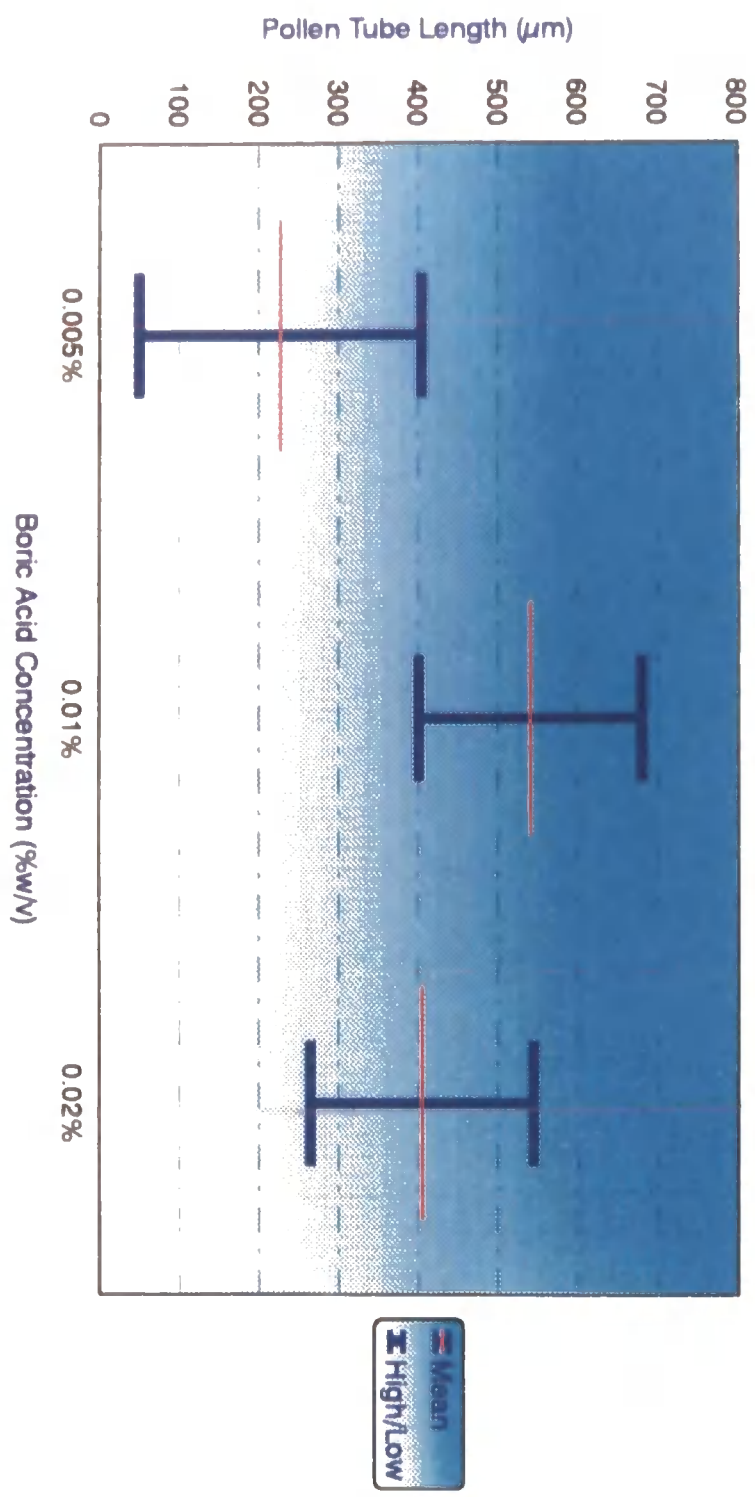


Figure (5.2): The effect of 5% sucrose supplemented with 0.01% boric acid, 100 ppm calcium nitrate, potassium nitrate and magnesium sulphate on pollen tube growth of *L. glandulifera*, 1 h in liquid PCM.

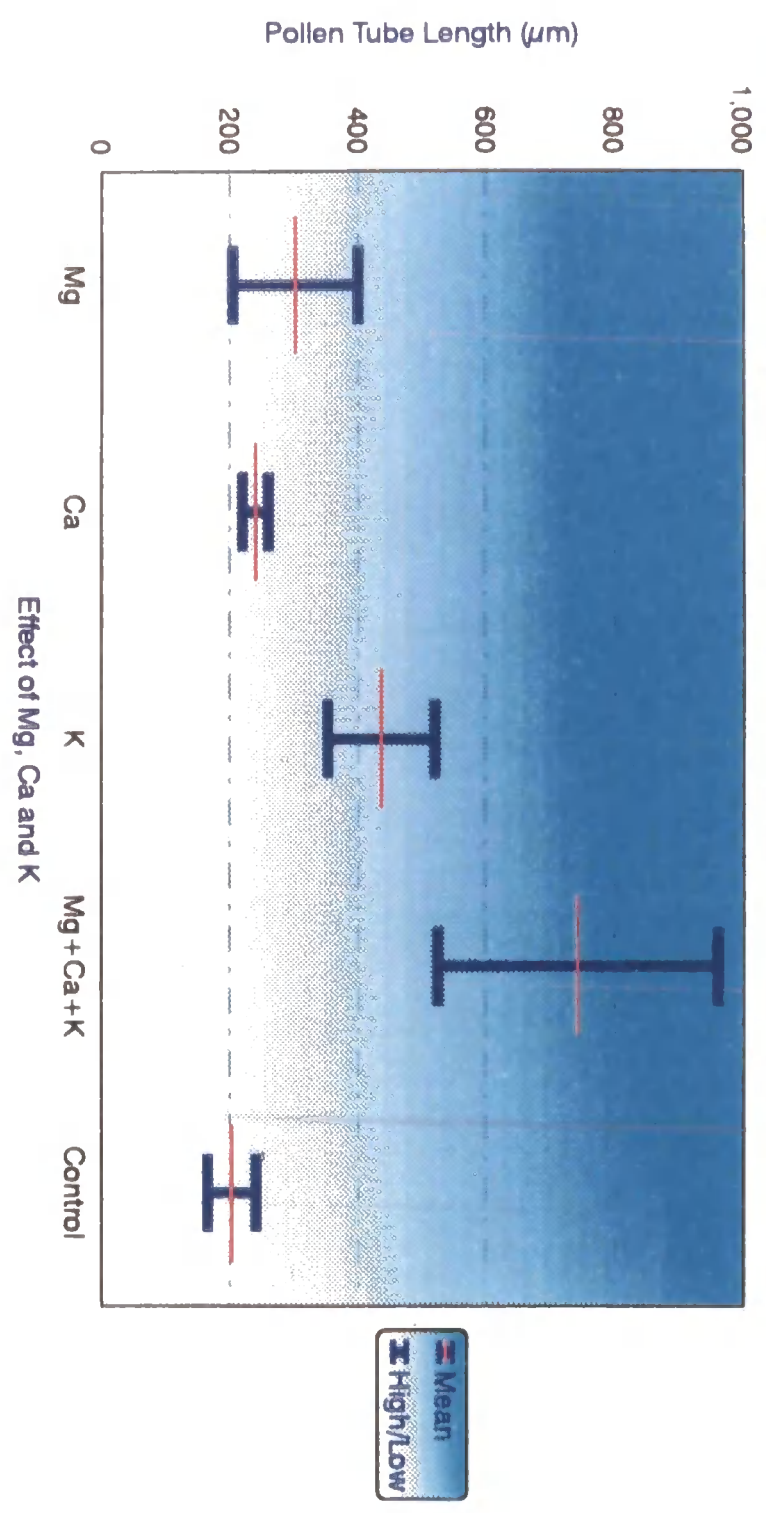
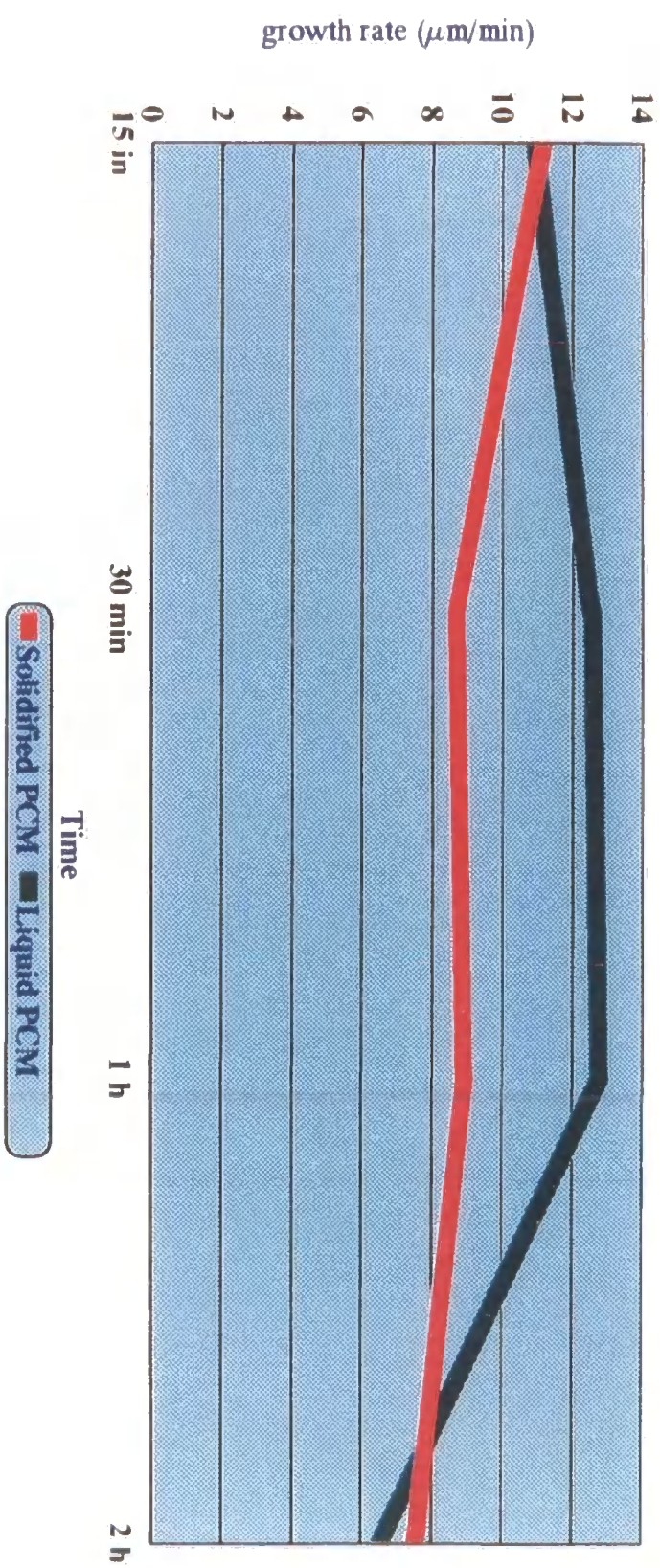




Figure (6): Pollen growth rate in *I. glandulifera* after incubation for 15 min - 2 h in liquid and solidified (1% agar) PCM



**Table (7) Pollen tube growth rate ( $\mu\text{m}/\text{min}$ ) of *I. glandulifera* after incubation in PCM supplemented with 1% (w/v) agar 15 min, 30 min, 1 h and 2 h at room temperature.**

<b>Level</b>	<b>Mean</b>	<b>StDev</b>	<b><math>\mu\text{m}/\text{min}</math></b>
<b>15 min</b>	167.80	36.40	11.18
<b>30 min</b>	261.00	75.10	8.70
<b>1 h</b>	535.50	45.60	8.92
<b>2 h</b>	899.00	104.60	7.49

### 3.1.2 Effect of temperature and humidity on pollen growth *in vitro*:

The effect of temperature on pollen tube length showed a significant difference ( $P=0.000$ ) as in Table (8) and Figure (7). Pollen tube lengths were greater at lower temperatures (8 °C) compared to room temperature; however, they also grew under lower temperatures down to 4 °C. Germination percentage was high both at 4-8 °C (86.3-90.6%) and at room temperature (24±1 °C) (82.1%). Despite the fact that pollen grew better under room temperature, they showed a higher rate of bursting compared to lower temperatures (4-8 °C) under which no pollen bursting was observed. The effect of humidity levels was also significant as shown in Table (9) and Figure (8) and pollen tube length increased with the increase of relative humidity (RH) from 0.0 to 55.0 to 75.0 to 92.0.

**Table (8) The effect of temperature on pollen growth of *I. glandulifera* in terms of tube length ( $\mu\text{m}$ ) and germination percentage after incubation for 45 min at 4 °C, 8 °C and room temperature (RT) in culture medium supplemented with 1% (w/v) agar.**

Source	DF	SS	MS	F	P
Factor	2	2495610	1247805	60.30	<0.001
Error	147	3042059	20694		
Total	149	5537669			

Pollen growth	4 °C	8 °C	RT
Tube length ( $\mu\text{m}$ )	624.8	802.2	487.1
$\mu\text{m}/\text{min}$	13.88	17.82	10.82
Germination (%)	86.3±3.2	90.6±3.6	82.1±3.7

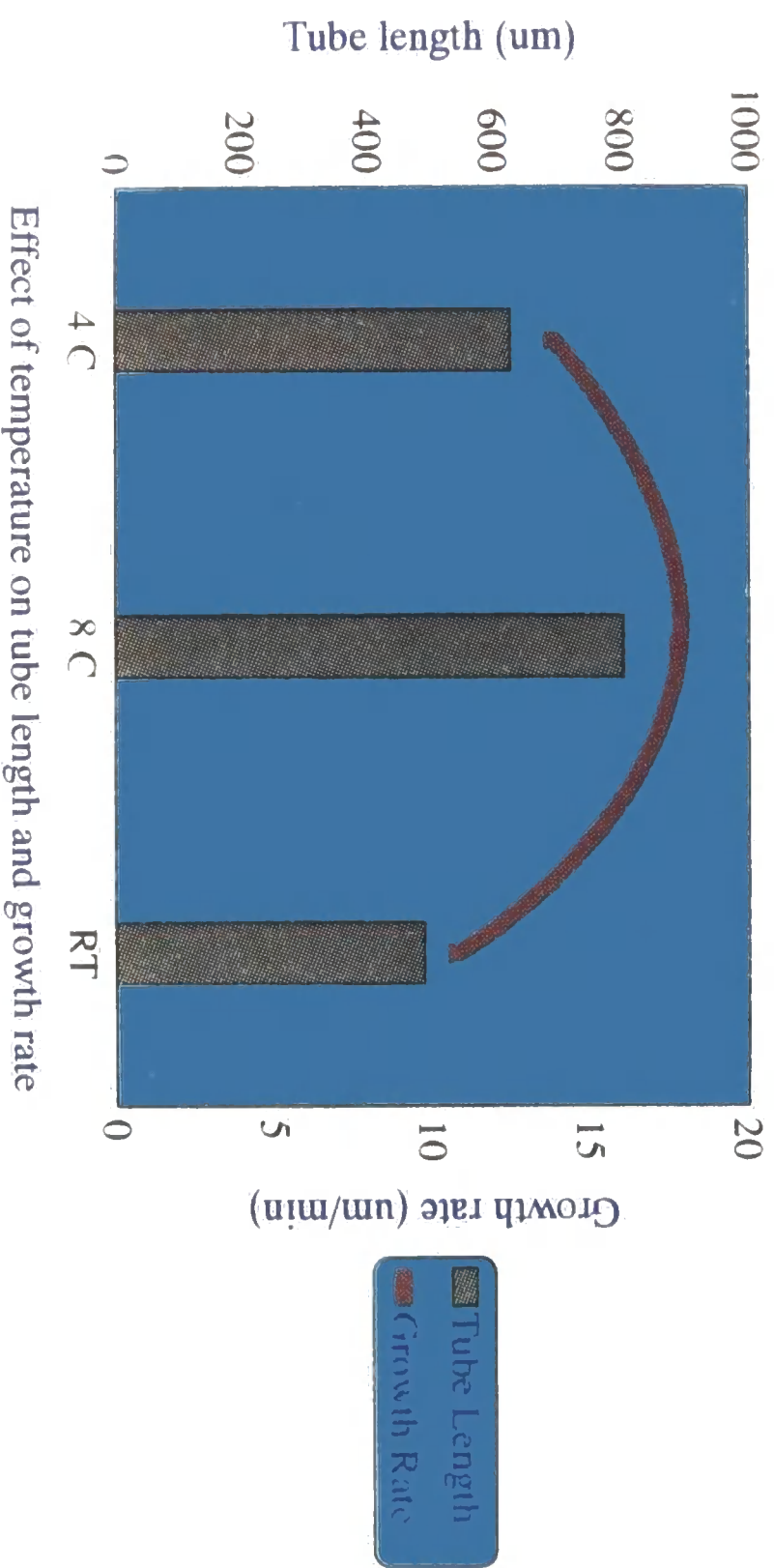
**Table (9) The effect of relative humidity (RH) on pollen growth of *I. glandulifera* in terms of tube length ( $\mu\text{m}$ ) and germination percentage after incubation for 90 min at room temperature (RT) using culture medium supplemented with 1% agar.**

Source	DF	SS	MS	F	P
Factor	3	581631	193877	20.19	0.00
Error	76	729656	9601		
Total	79	1311287			

Pollen growth	RH=0	RH=55.0	RH=75.0	RH=92.5
Tube length ( $\mu\text{m}$ )	252.45	449.90	532.00	576.05
$\mu\text{m}/\text{min}$	3.91	4.99	5.91	6.40
Germination (%)	87.87 $\pm$ 5.8	90.1 $\pm$ 3.8	84.5 $\pm$ 5.3	85.6 $\pm$ 2.3

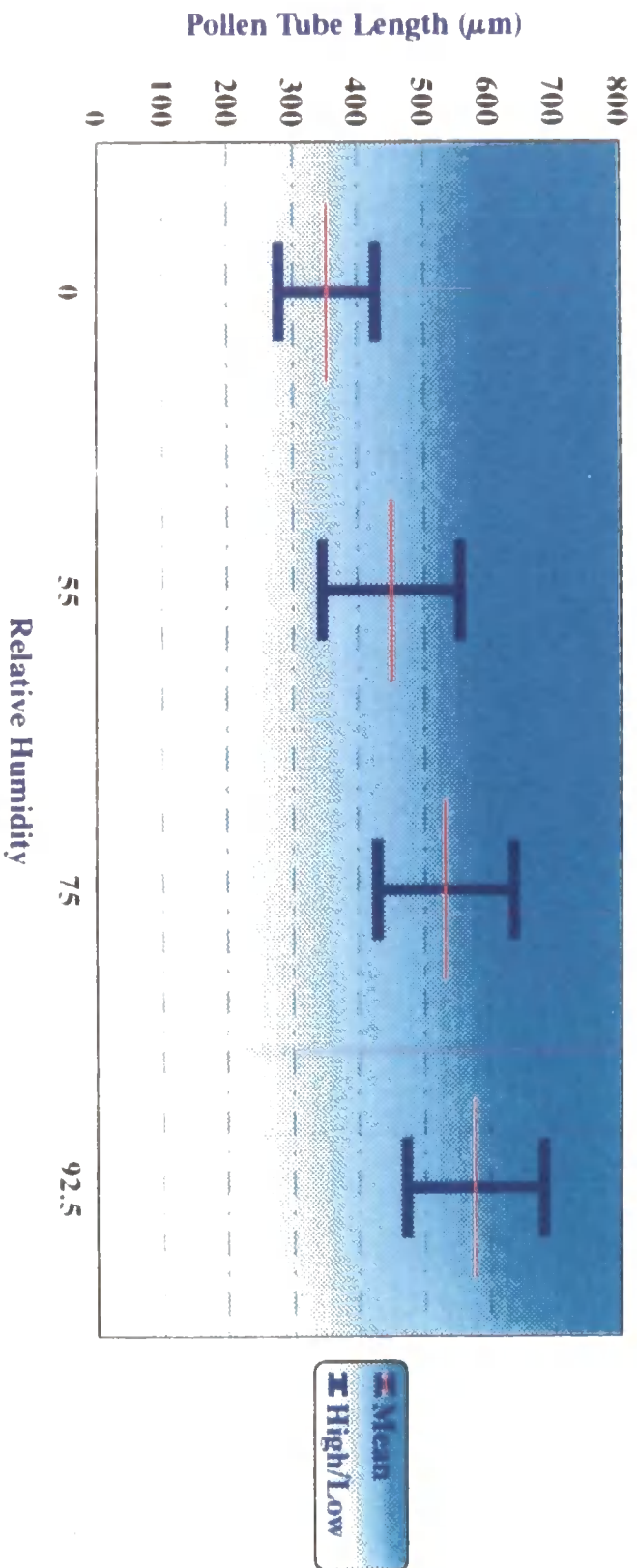


Figure (7): Tube length and growth rate in *I. glandulifera* pollen after incubation for 45 min at 4 °C, 8 °C and room temperature (24±1) in culture medium supplemented with 1% agar.



Effect of temperature on tube length and growth rate

Figure (8): The effect of humidity on pollen tube growth of *I. glanduifera* after incubation for 90 min at room temperature in culture medium supplemented by 1% agar



### **3.1.3 Assessment of pollen viability at different temperature and humidity conditions:**

Both concentrations of CFW (0.1% and 1% w/v) gave similar effect on fluorescence intensity as shown in Table (10). CFW was found to be non-toxic up to 1% (w/v) as pollen readily germinated in a medium containing CFW at this concentration. Therefore a concentration of 0.1% (w/v) CFW was selected for assessment of pollen viability after incubation for 24 h under different temperature conditions. Better pollen growth in terms of tube length was achieved at low temperatures (4 °C and 8 °C) compared to room temperature (Figure 7); however, at high temperature (60 °C) high bursting and no germination resulted, which agrees with the findings by Vasil and Bose (1959) who reported pollen bursting at temperatures higher than 35 °C.

Treatment of pollen batches with 1% (w/v) CFW for 5 min showed a significant variation in fluorescence intensity between pollen incubated at different temperatures for 24 h as appears in Table (11). Higher fluorescence intensity was shown by pollen incubated at 4 °C compared to other temperature levels; however, the least fluorescence intensity resulted after incubation at 60 °C. This means that there is a decline in fluorescence intensity due to treatment with CFW with the increase in incubation temperature as appears in Figure (10).

The reaction to the FCR test by *I. glandulifera* pollen was different from the previous reaction to treatment with CFW. Fluorescence intensity significantly increased with the increase in incubation temperature; however, it was far lower than the reaction shown by pollen immediately after anther dehiscence which gave the highest result as shown in Figure (11).

The FCR test on fresh pollen from *I. glandulifera*, *N. tabacum* and *B. napus* showed a nonsignificant difference between the three species in their fluorescence due to retention of fluorescein (Figure 9) which could be correlated with pollen surface area.

Figure (9): Fluorescence intensity (FI) of fresh pollen from *I. glandulifera*, *Nicotiana tabacum* and *Brassica napus* after treatment with FDA for 5 min

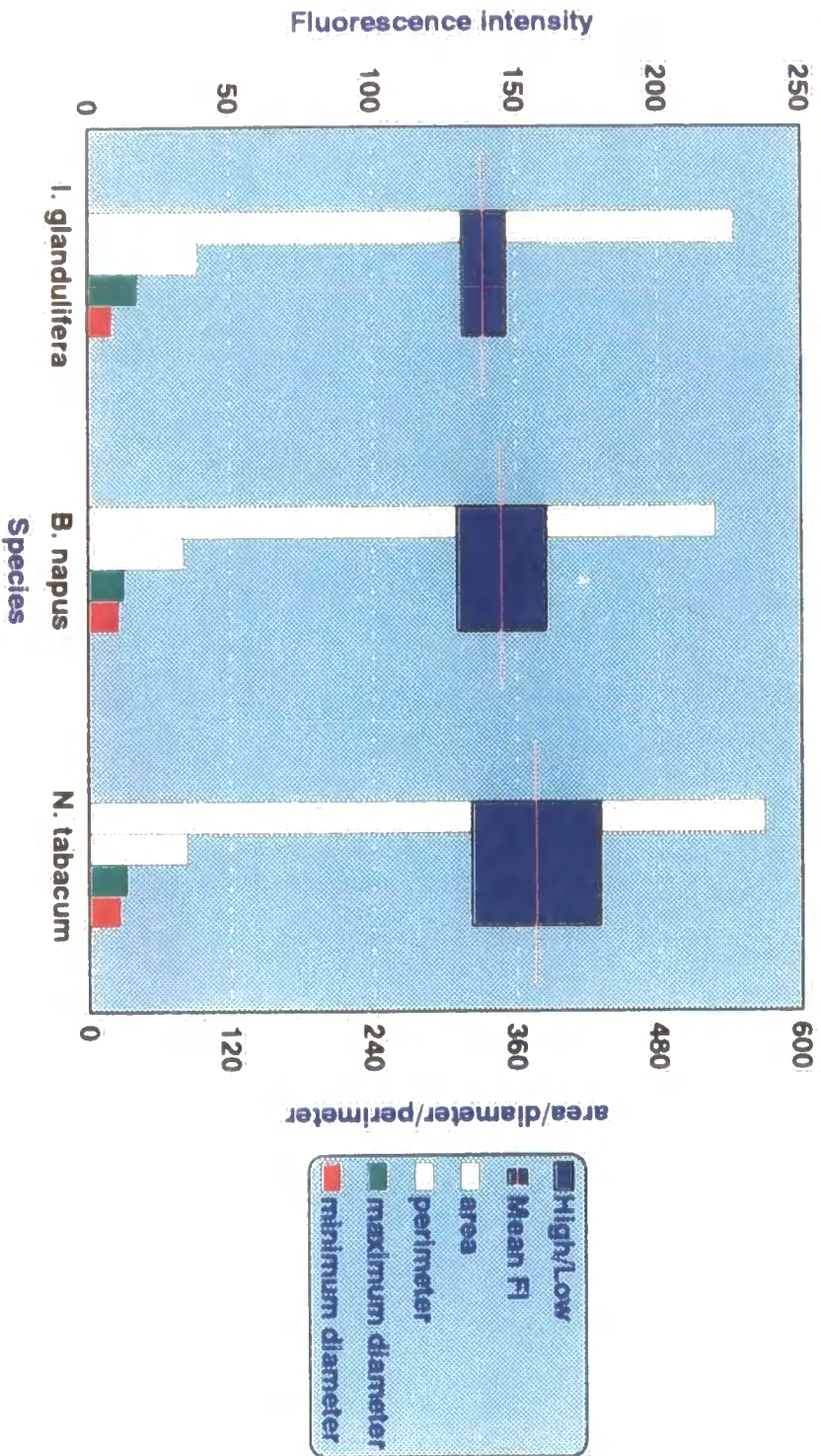


Figure (10): Fluorescence intensity after treatment by CFW of pollen preincubated at 4 °C, 8 °C and room temperature (RT) and 60 °C for 24 h.

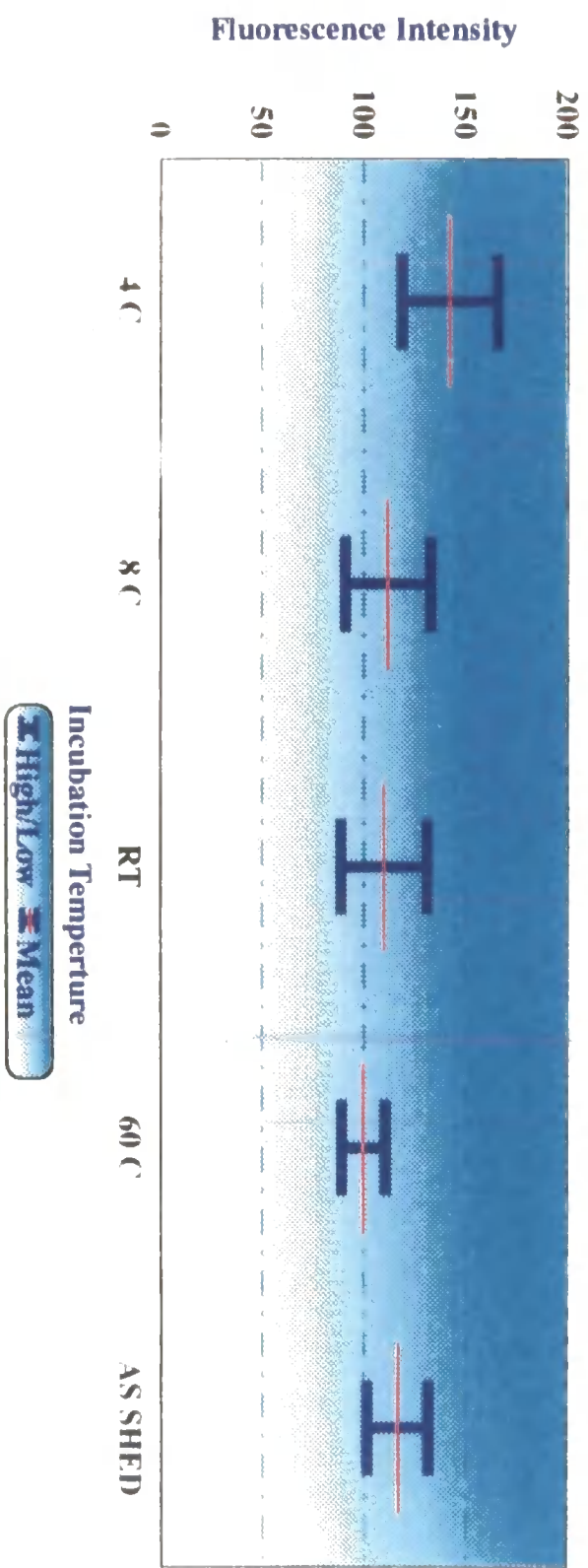
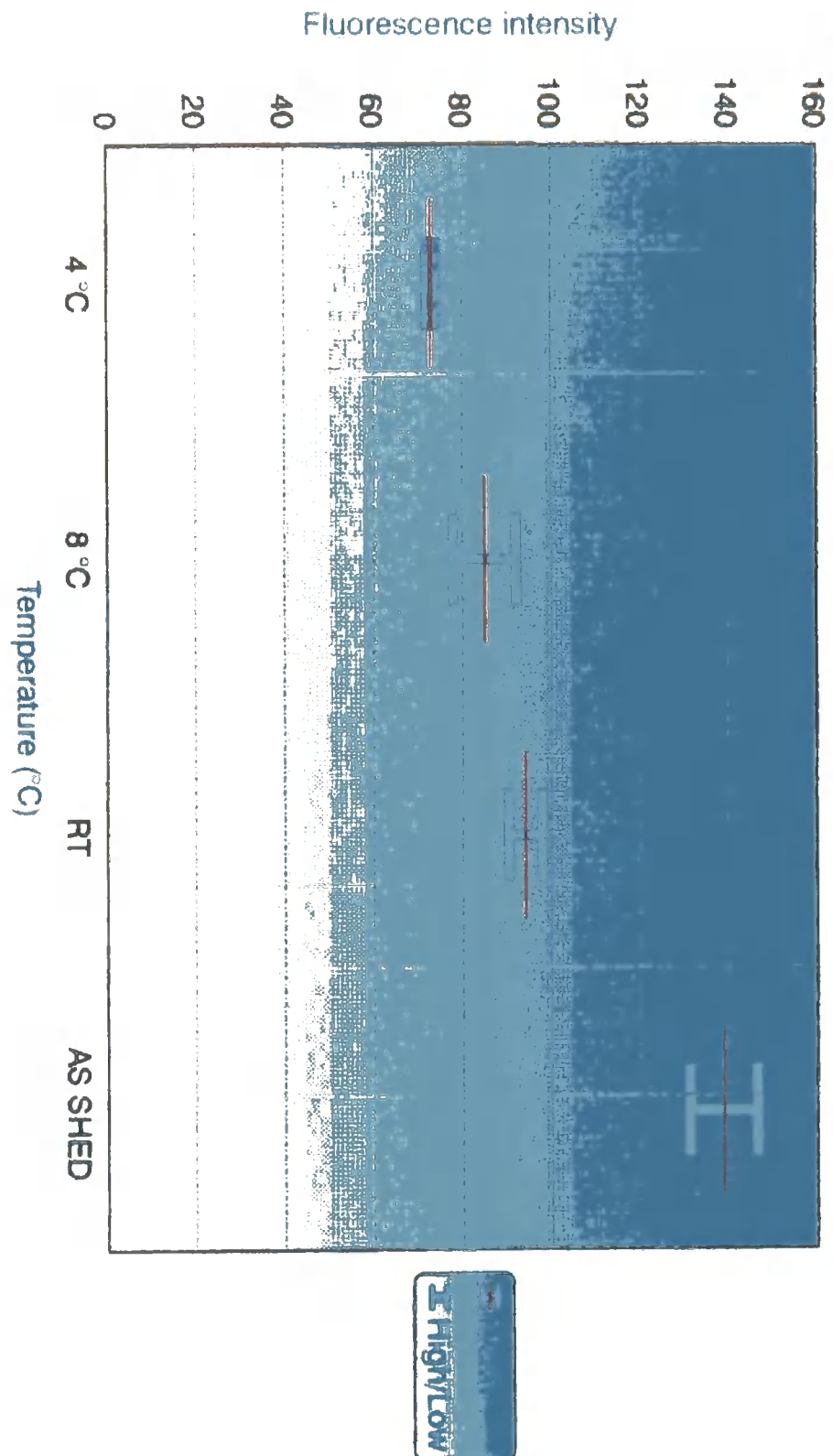




Figure (11): Fluorescence intensity of fresh pollen from *L. glandulifera* incubated for 24 h at 4 °C, 8 °C and room temperature (RT) after treatment with FDA for 5 min.



**Table (10)** Fluorescence intensity of fresh *Impatiens glandulifera* pollen after incubation in CFW (0.1-1% w/v) for 5 min.

Intensity	0.1% (w/v)	1% (w/v)
Minimum	81.00	81.00
Maximum	90.92	99.86
Mean	83.30	84.06
Median	82.60	92.40
Variance	4.691	23.15
StDev	2.166	4.811
Count	219	411

**Table (11)** Analysis of variance of fluorescence intensity of fresh *Impatiens glandulifera* pollen after incubation in CFW (1% w/v) for 5 min as measured from pollen incubated at 4 °C, 8 °C, room temperature (RT) and 60 °C for 24 h.

Source	DF	SS	MS	F
Factor	3	98584	32861	82.79
Error	396	157182	397	
Total	399	255766		

Level	Mean	StDev
4 °C	141.97	23.96
8 °C	115.78	20.94
RT	109.54	21.37
60 °C	99.52	10.89



#### **3.1.4 Assessment of pollen developmental state and monitoring of pollen nuclei:**

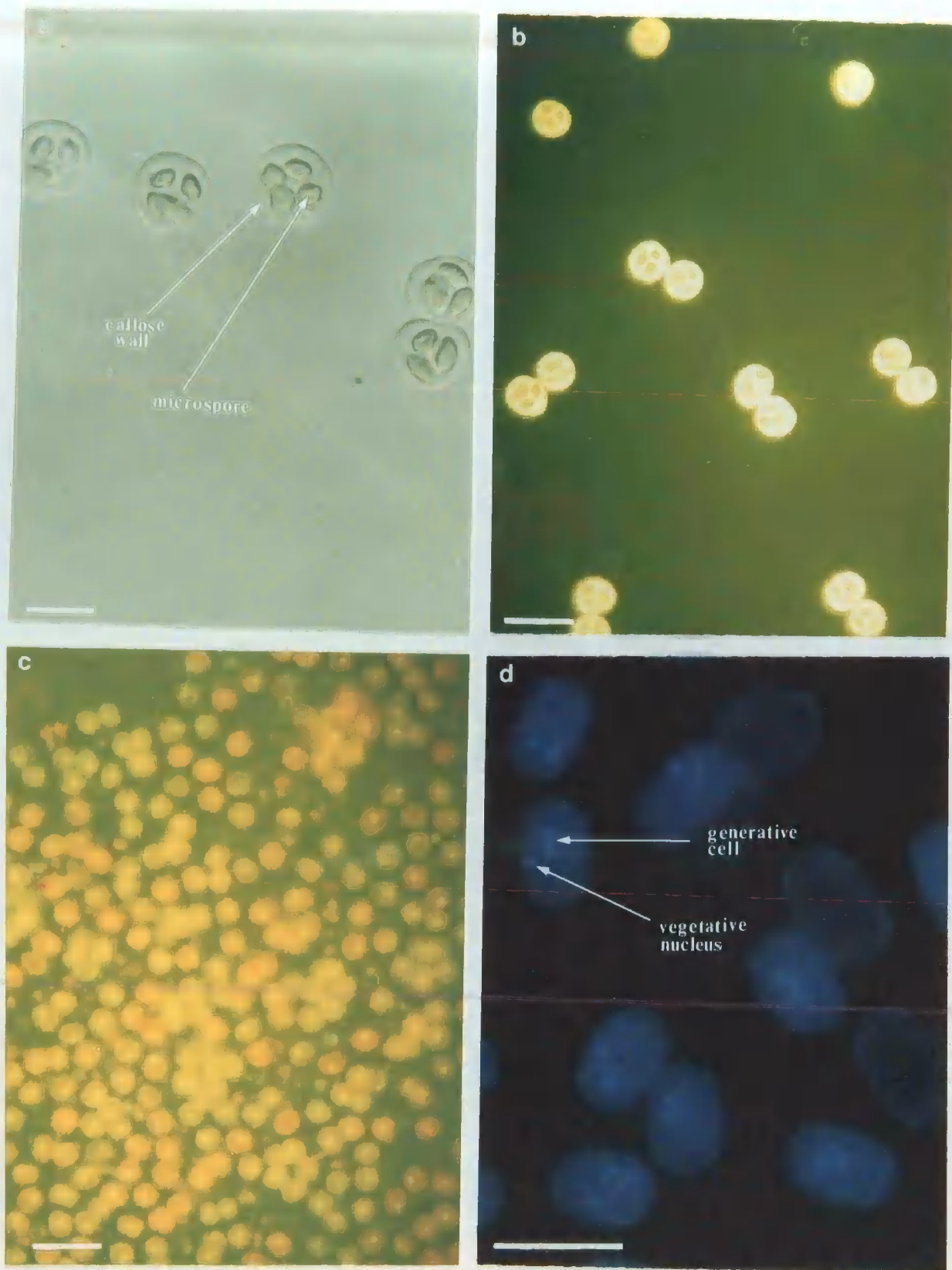
In the first stage of floral bud development, all microspores were linked with each other by a callose wall, and no exine had developed. The second stage showed the end of meiosis and formation of tetrads (Figure 12a). Tetrads then began to break up to single microspores. Later, they started to deposit sporopollenin. As they attained maturity, they developed germination pores.

At the end of meiosis, when buds pre-treated with Feulgen staining stage were treated with aniline blue, they showed a strong fluorescence of callose enclosing microspores (Figure 12b). Treatment with acridine orange (Figure 12c) at this stage showed both bright yellow (DNA) and red fluorescence (nucleus RNA). Staining with DAPI revealed uninucleate tetrads, and two nuclei (tube cell and generative cell) after break up of tetrads to single microspores (Figure 12d). In some species, the generative cell undergoes a mitotic division within the pollen grain, forming two sperm cells (Mascarenhas, 1989). In others, as is the case with *I. glandulifera*; however, the generative cell gametes complete their division during the growth of the pollen tube in the style.

#### **3.1.5 Loading of fluorochromes into pollen by Plasmolysis/endocytosis:**

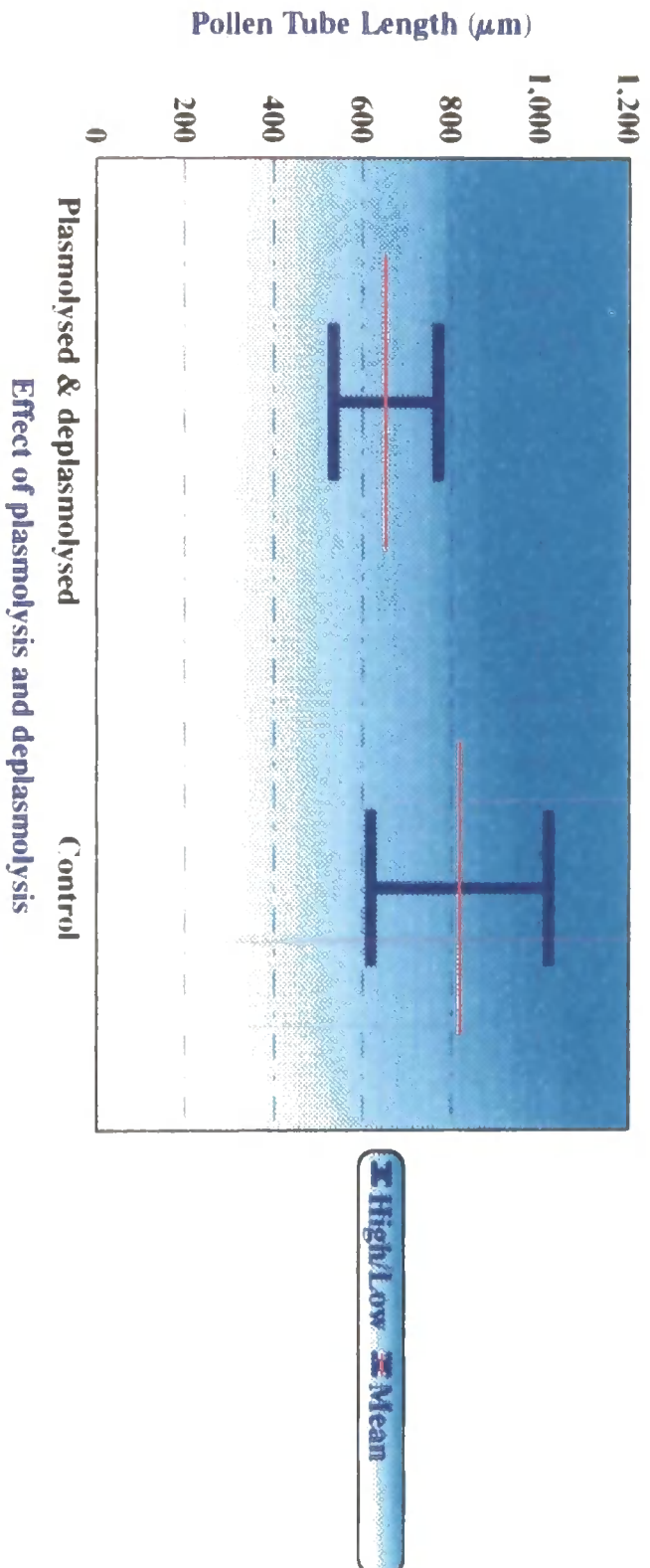
The effect of plasmolysis on pollen tube growth was shown in Figures 13 & 14 which reveals a non-significant effect of plasmolysis on pollen tube final length after subsequent rehydration and growth. Pollen loaded with LY-CH after plasmolysis in the presence of the probe and deplasmolysis are shown containing vesicles in Figure 15a. Pollen nuclei fluoresced bright yellow under blue excitation as shown in microtome sections in Figure (15b), due to accumulation of vesicles around nuclei.

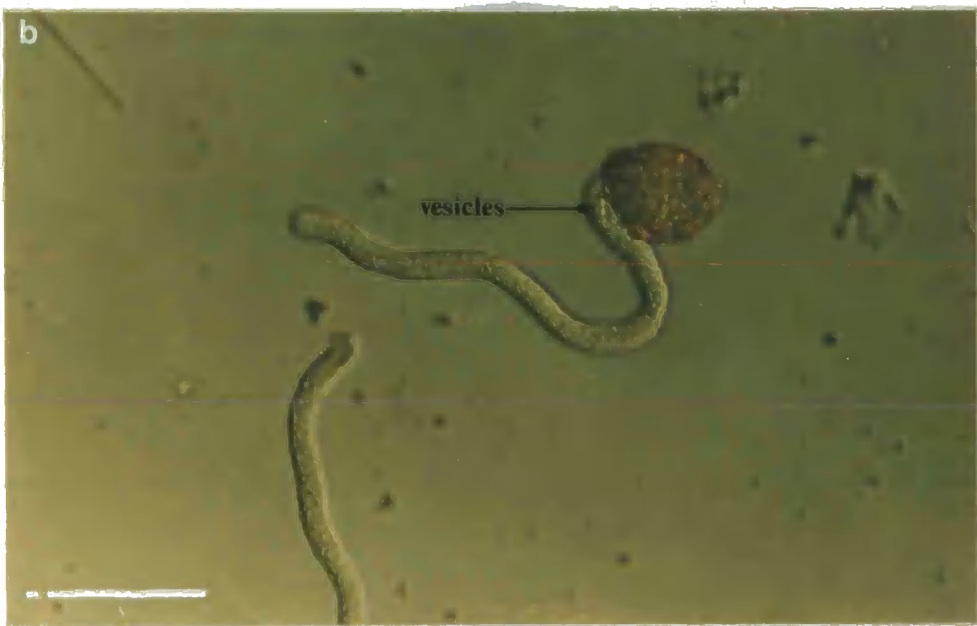
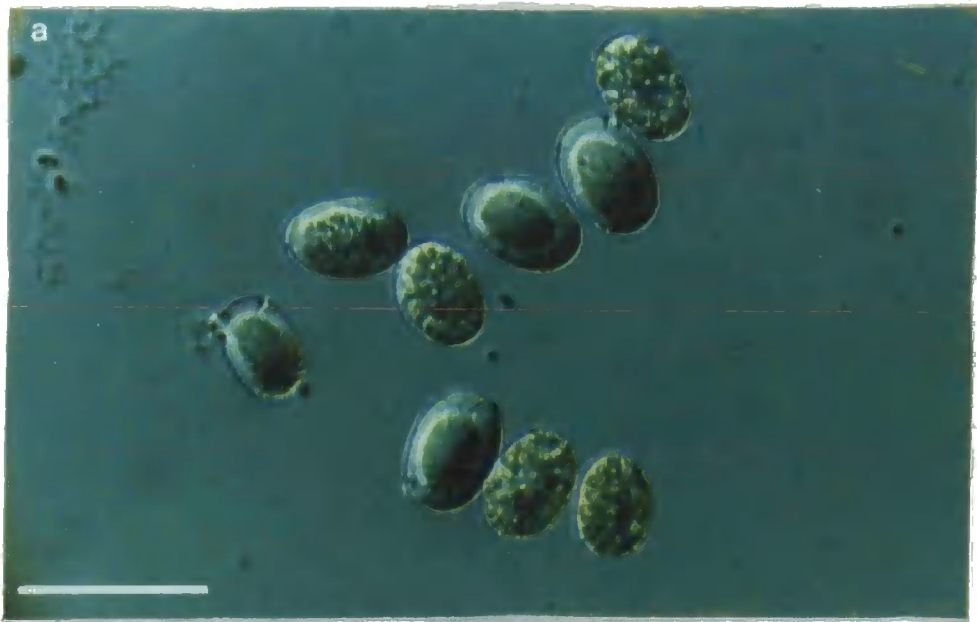
Uptake of fluorescein by pollen was also evident (Figure 15c) after plasmolysis and deplasmolysis. Strong fluorescence resulted under blue filter excitation. Pollen germinated in all cases, indicating that the probes used were non-cytotoxic. These results prove that xenobiotics could be loaded into pollen by plasmolysis/endocytosis. Microscopic examination of pollen from *I. glandulifera* immersed in distilled water (normal osmotic conditions) using the Nomarski DIC revealed no endocytic vesicles, however, pollen bursting was quite evident.



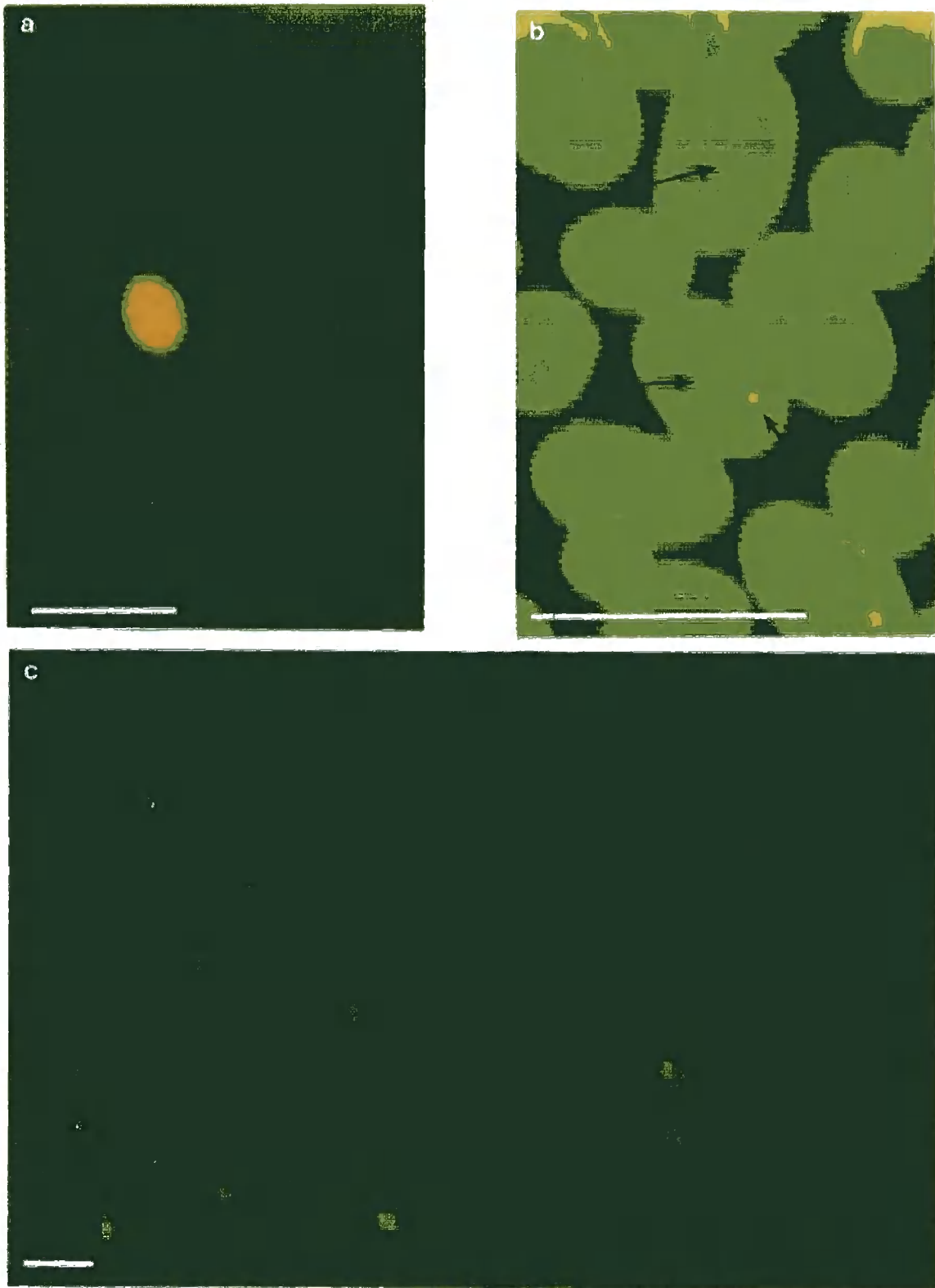
**Figure (12):** Assessment of pollen state and monitoring of pollen nuclei in *I. glandulifera*. (a) Tetrads at the end of meiosis. (b) Tetrads showing callose wall fluorescence after treatment with aniline blue. (c) Nuclei fluorescing after treatment with acridine orange (microspores separated from tetrads). (d) Mature pollen grain showing vegetative and generative nuclei after staining with DAPI.

Figure (13): The effect of plasmolysis and deplasmolysis on growth of *I. glandulifera* pollen.





**Figure (14): (a) Pollen from *I. glandulifera* after plasmolysis in 1 M manitol for 1 h. (b) plasmolysed and deplasmolysed pollen germinated in PCM 1 h after incubation, showing vesicles in pollen tube. (Bars=50 $\mu$ m).**

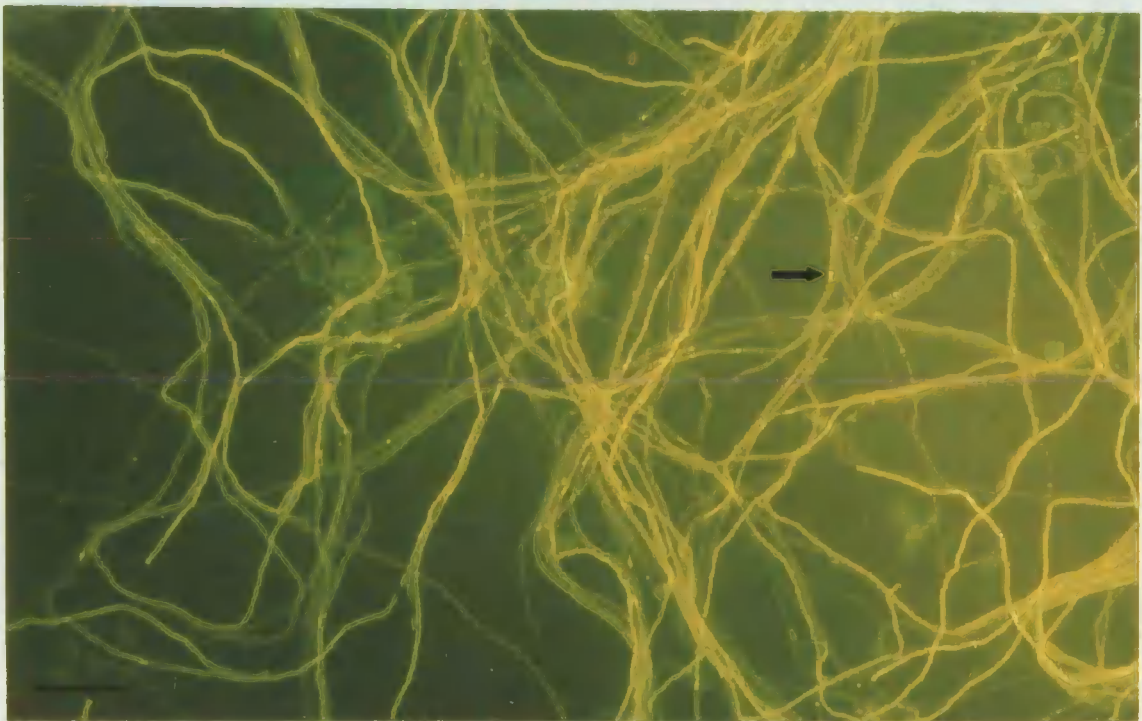


**Figure (15): (a) Pollen from *L. glandulifera* after plasmolysis in 1 M manitol supplemented by 5 mg ml<sup>-1</sup> lucifer yellow, and deplasmolysis for 30 min, showing fluorescent vesicles. (b) microtome section after embedding in LR White showing nuclei. (c) pollen germinating after uptake of fluorescein showing probe in pollen tubes. (Bars = 50  $\mu$ m).**

### 3.1.6 **Plasmolysis/deplasmolysis of pollen, *in vitro* pollination and identification of pollen tubes:**

Pollen tubes were clearly identified under both *in vivo* and *in vitro* growth conditions by aniline blue staining of callose deposits (see protocol 2.2.8). Callose plugs fluoresced blue under BV filter excitation. Three different shapes of callose plugs were identified in *I. glandulifera* pollen germinating *in vivo* as shown in Figure (17). Distance before callose plugs were laid down was measured for the first and second callose plugs in *I. glandulifera* and *N. tabacum* (Figure 16, 18). The results showed that in *I. glandulifera* the distance to the first callose plug is shorter than to the second callose plug. However, a constant distance between callose plugs was shown by *N. tabacum*, making the number of callose plugs in this species a sensitive indicator of pollen tube growth rate (Snow and Spira, 1991).





**Figure (16): Callose plugs in *Nicotiana tabacum* (top) and *Impatiens glandulifera* (bottom) 6 h after incubation in PCM supplemented with 1% agar and treatment with decolourised aniline blue (bars=50 $\mu$ m).**



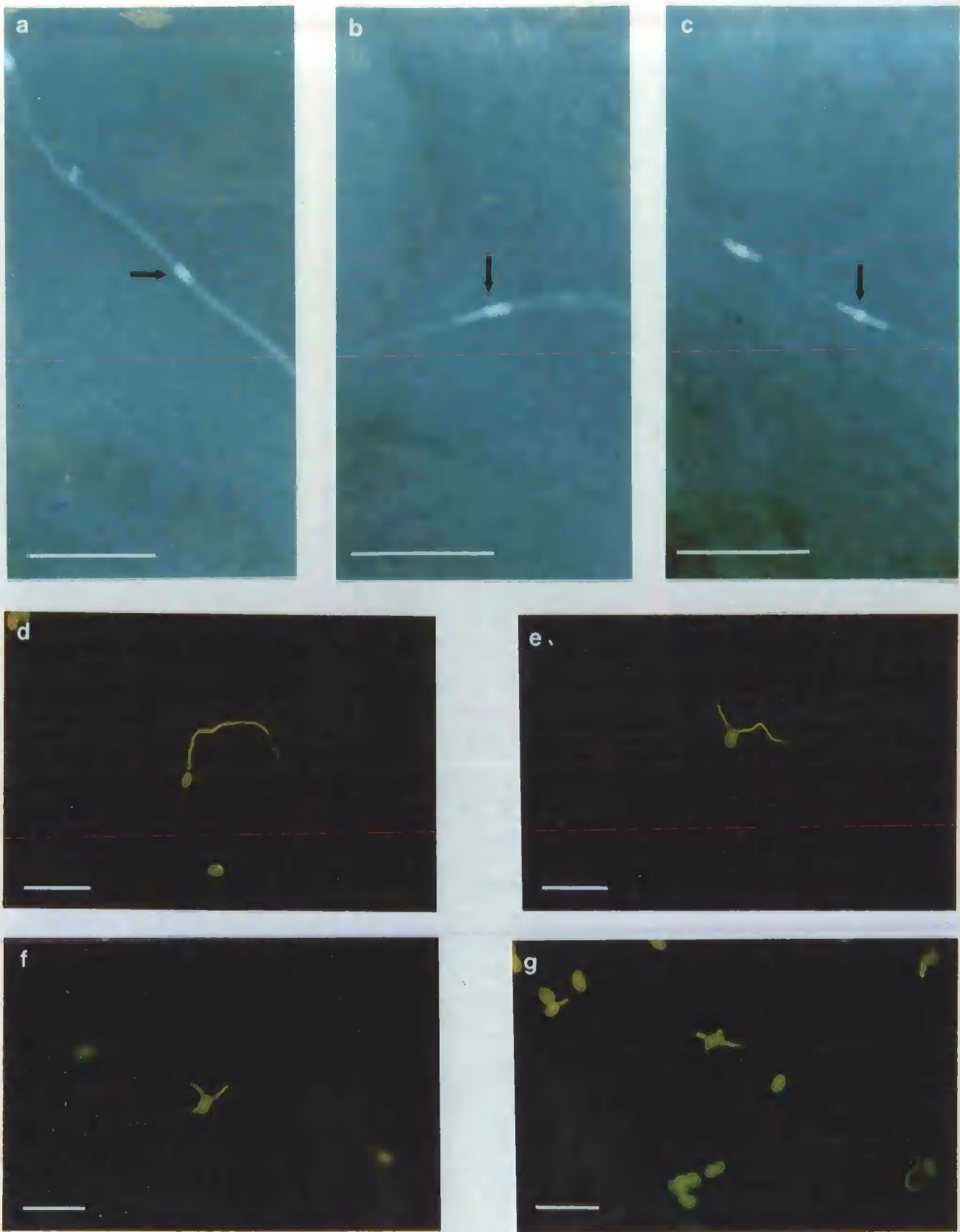
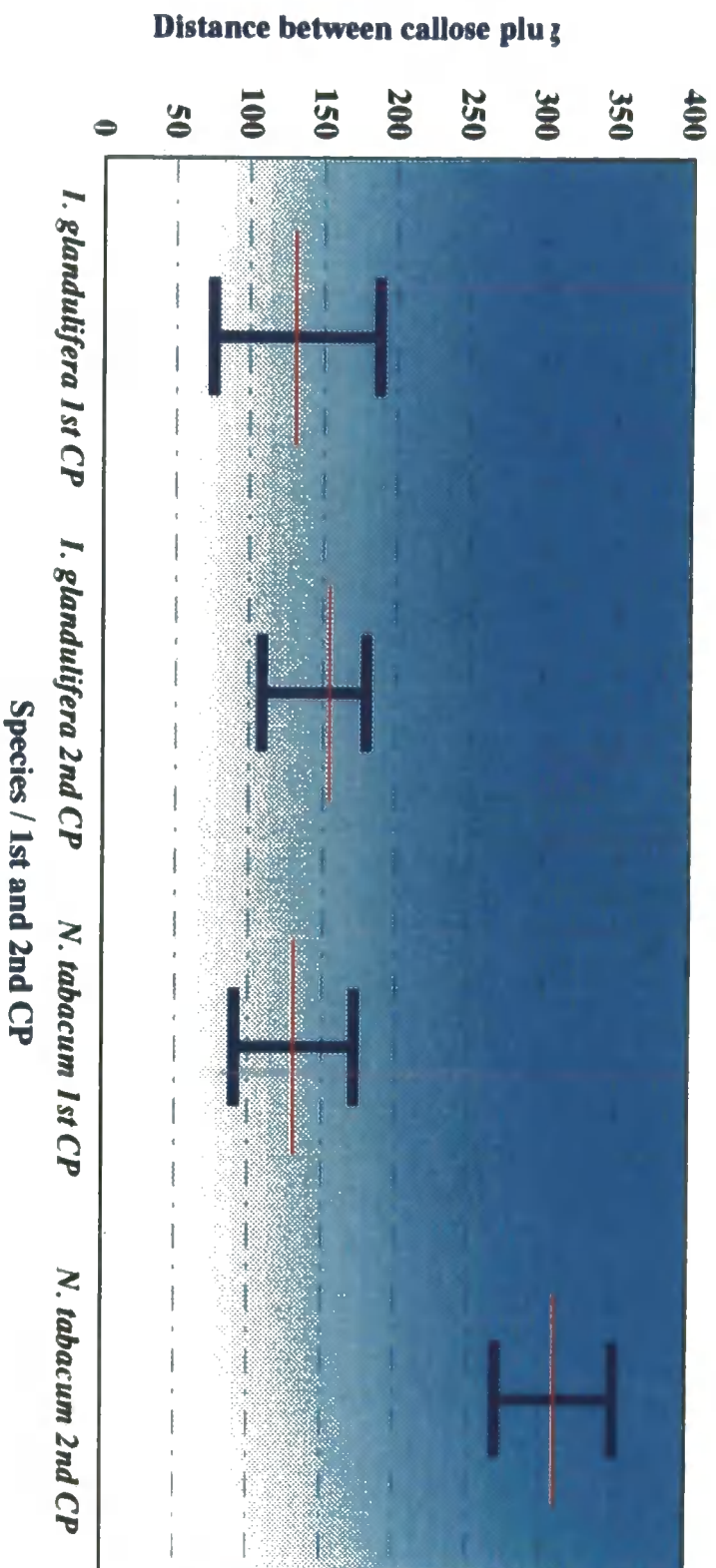


Figure (17): (a-c) *I. glandulifera* pollen tubes identified in vivo after treatment with aniline blue showing three types of callose plugs. (d-g) one to four pollen tubes emerging from single pollen grains.

Figure (18): Distance to first and second callose plugs (CP) of *I. glanduifera* and *N. tabacum* after incubation in PCM supplemented with 1% agar.



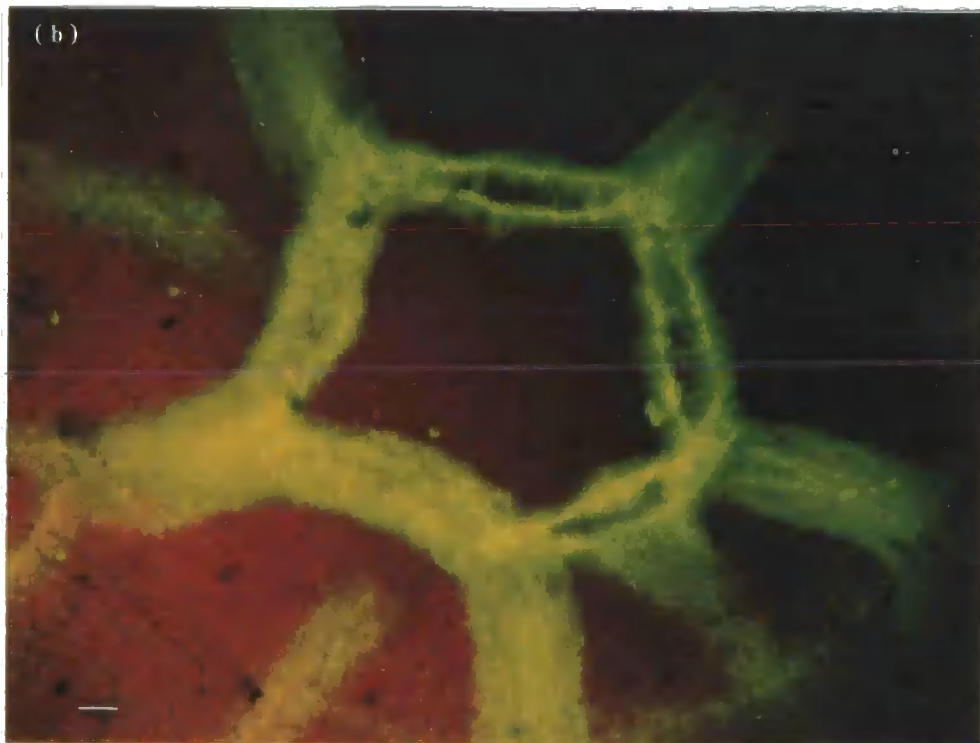
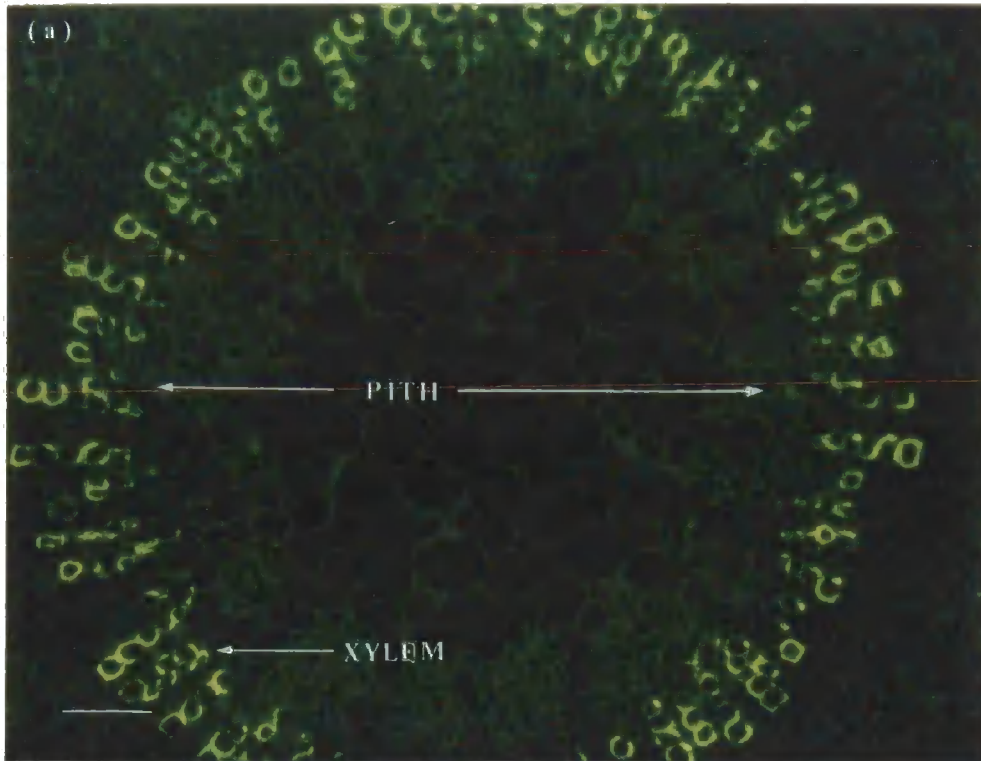
### 3.2 Loading of fluorochromes into plant cells via the vascular system:

It is evident from Figures (19a and 19b) that LY-CH introduced via the pedicel was transported apoplastically through the xylem cells up to the ovules of *Nicotiana tabacum* (Figure 20a) and *I. glandulifera* (Figures 20b and 20c), as shown by the yellow fluorescence. The results obtained in Figure (19b) shows uptake of LY-CH via vascular routes branching from the central vascular system of the pedicel towards floral appendages. However, as shown in Figure (20a) the probe accumulated in the funiculus of the former species without been taken into the ovules, compared to the latter species where ovules fluoresced as a result of accumulation of the probe. Traces of LY-CH were also seen in the style and stigma cells of *N. tabacum* after loading of the probe via the pedicel.

CFW was also transported apoplastically, as evident from the blue fluorescence of all cell walls of *Brassica napus* pedicel (Figure 21a). At the funiculus (Figure 21b), however, the probe has taken two routes around the ES. These routes represent the vascular strands which develop during the formation of the funiculus and end commonly at the chalazal part of the nucellus, as described by Willemse (1992).

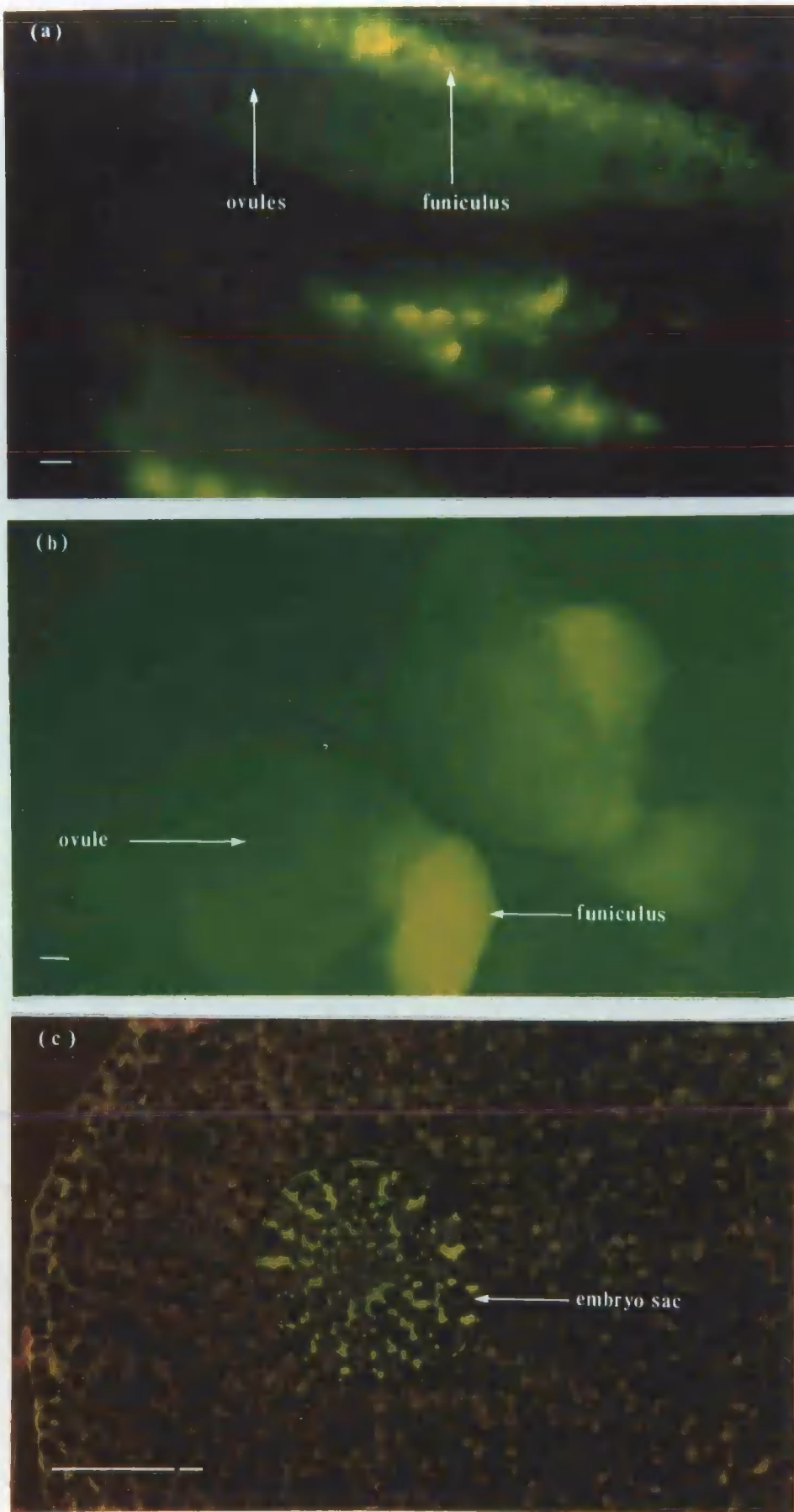
The uptake of fluorescein via the *Brassica napus* pedicel (Figure 22b) resulted in accumulation of the probe in the funiculus. Uptake of LY-CH (Figure 22a) showed strong accumulation of LY-CH in both the placenta and the funiculus.

Fluorescein loaded via the pedicel of *Impatiens glandulifera* pre-anther opening was taken up by pollen . When this pollen was germinated, the probe was clearly visible in the growing pollen tubes (Figure 23). It can also be observed in Figure (23), that the probe did not enter the pollen vacuole which appeared as a dark area in the centre of the pollen.

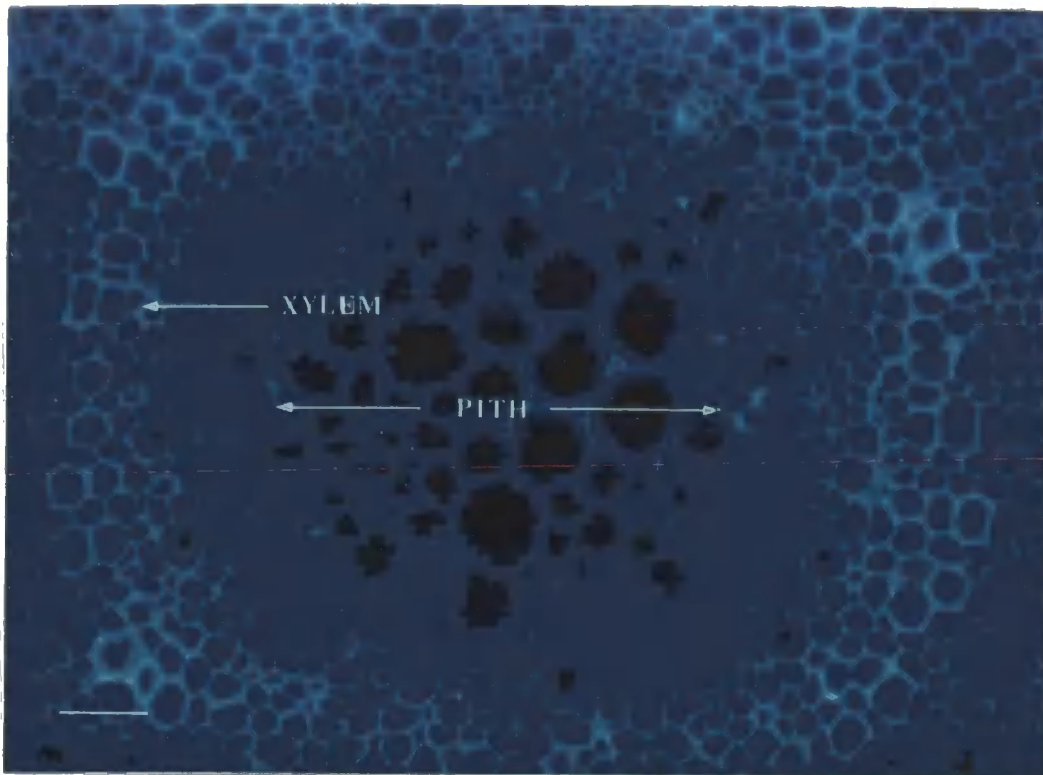


**Figure (19): (a) 12  $\mu\text{m}$  thick transverse section of pedicle and (b) 'free hand' transverse section at ovary base of *Nicotiana tabacum*, 2 h after incubation in 5  $\text{mg ml}^{-1}$  Lucifer Yellow CH, viewed under blue filter excitation. (bars = 50  $\mu\text{m}$ ).**

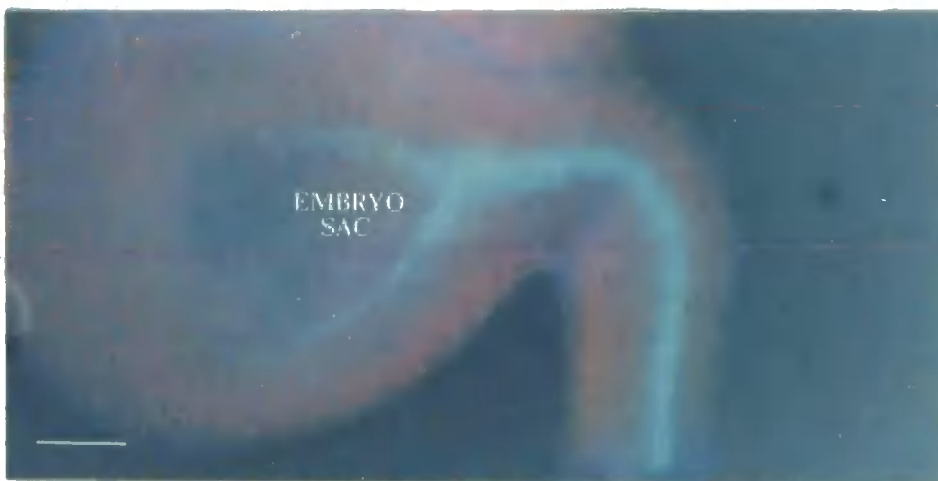




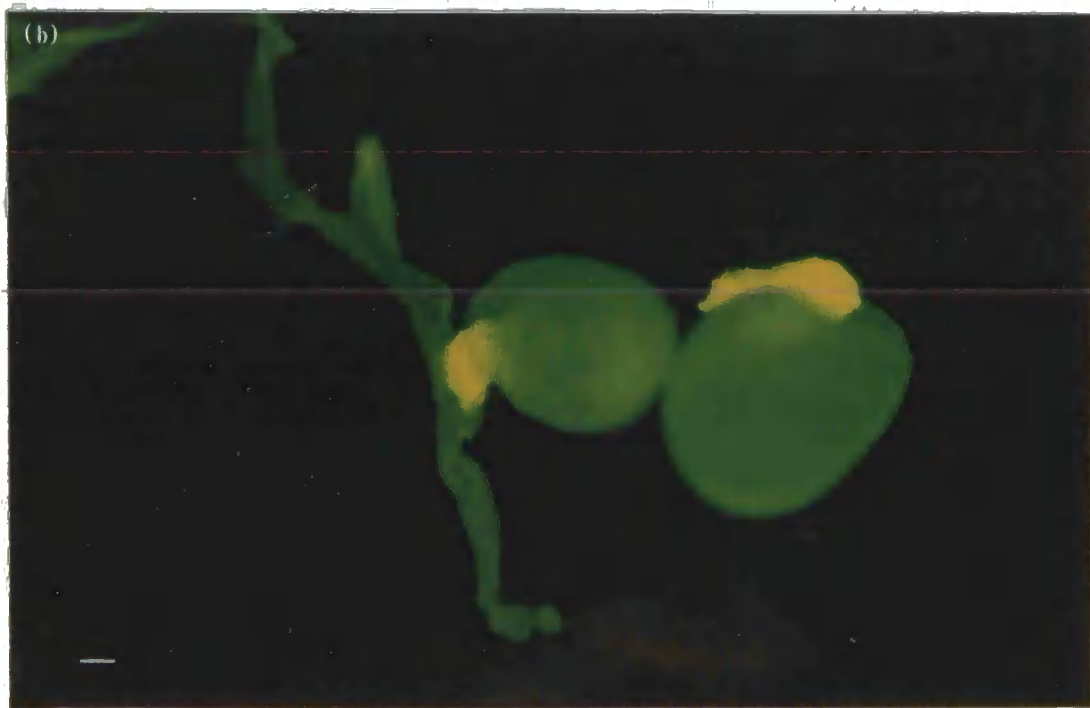
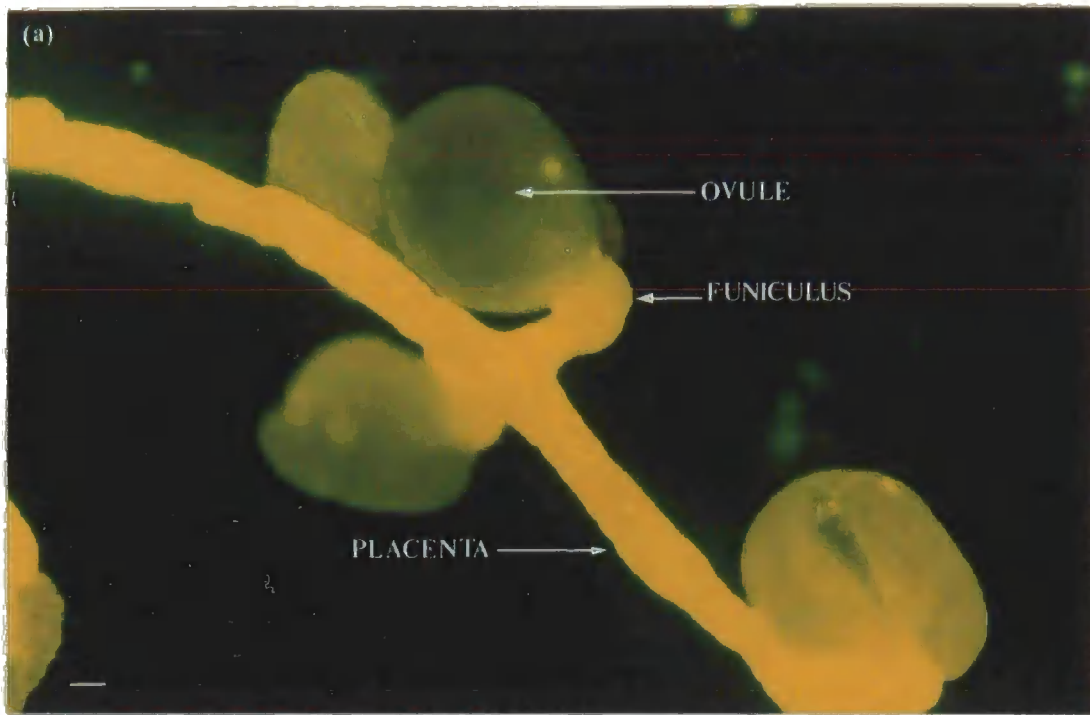
**Figure (20): Uptake of Lucifer Yellow CH via pedicle as shown by funiculus of (a) *Nicotiana tabacum* and (b) *Impatiens glandulifera*. (c) 12 μm thick section of *I. glandulifera* ovule, viewed under blue filter excitation. (bars = 50 μm).**



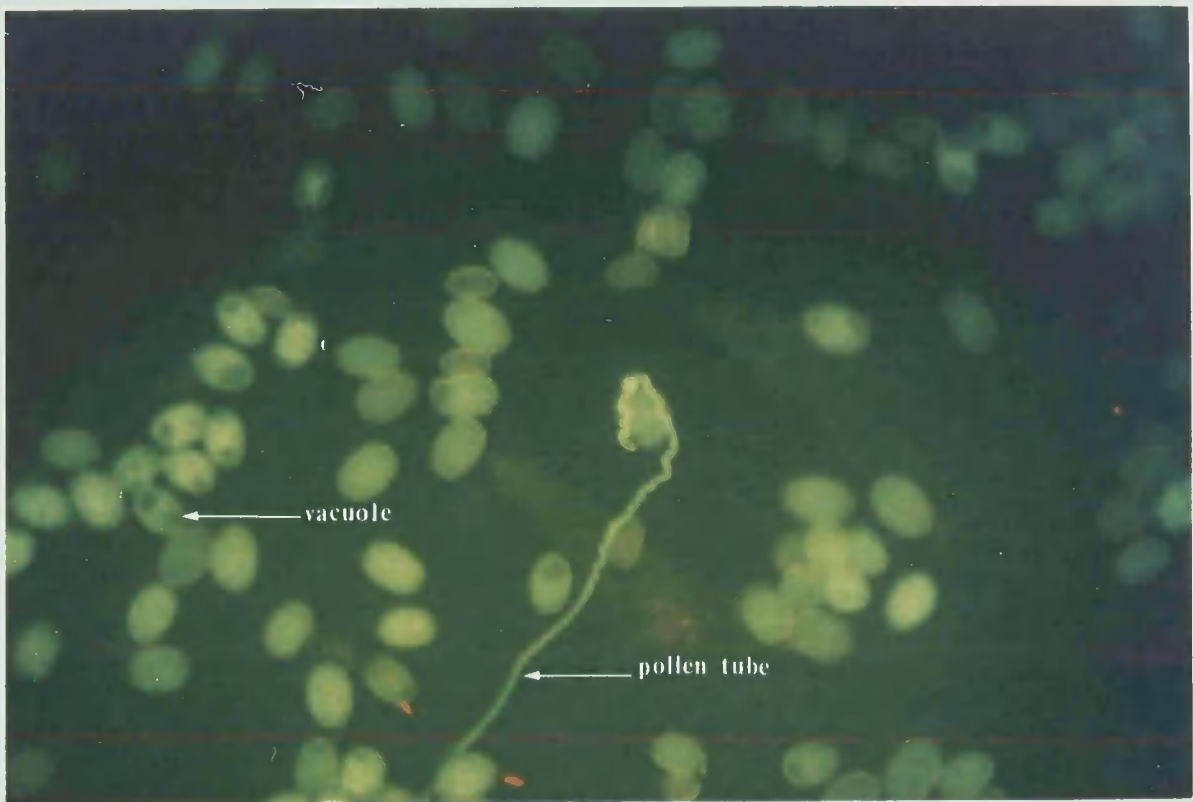
**Figure (21a):** Transverse section of *Brassica napus* pedicle after incubation in CFW for 2 h, viewed under UV excitation filter. (Bars = 50  $\mu\text{m}$ ).



**Figure (21b):** *Brassica napus* ovule after incubation of pedicle in CFW for 2 h, viewed under UV excitation filter. (Bars = 50  $\mu\text{m}$ ).



**Figure (22):** Relative mobility of LY-CH and fluorescein in *Brassica napus* ovary after incubation of pedicel for 2 h in (a) LY-CH and (b) fluorescein, photographs were taken under blue light excitation filter (bars = 50  $\mu\text{m}$ ).



**Figure (23):** Loading of fluorescein in *I. glandulifera* pollen via the pedicel pre-anther opening, after immersion of the pedicel for 1 h in 1 mg ml<sup>-1</sup> solution of the fluorescent probe. Pollen germination took place in PCM. (Bars = 50  $\mu$ m).



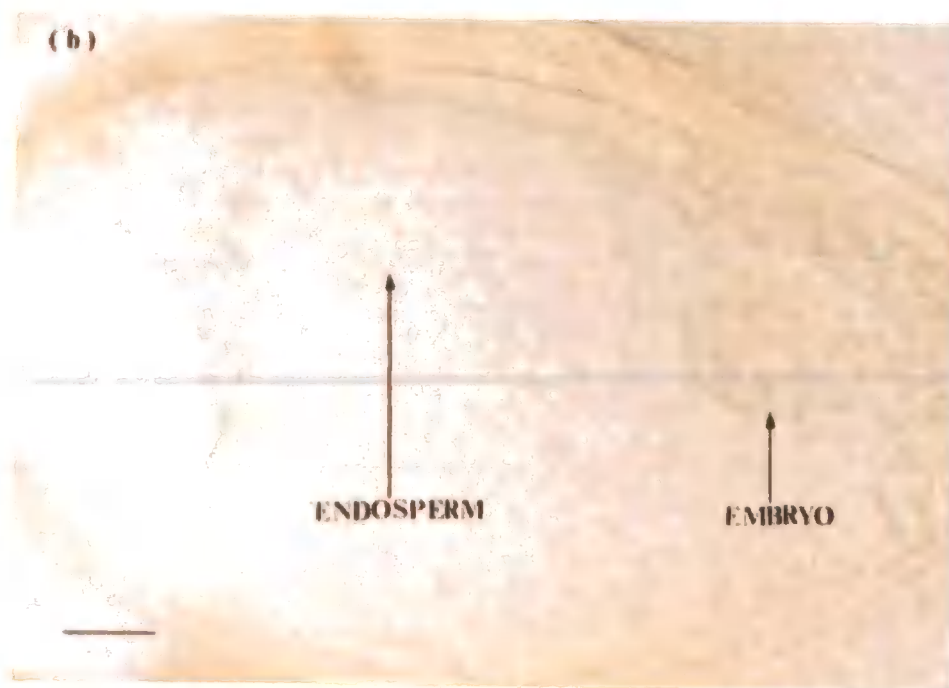
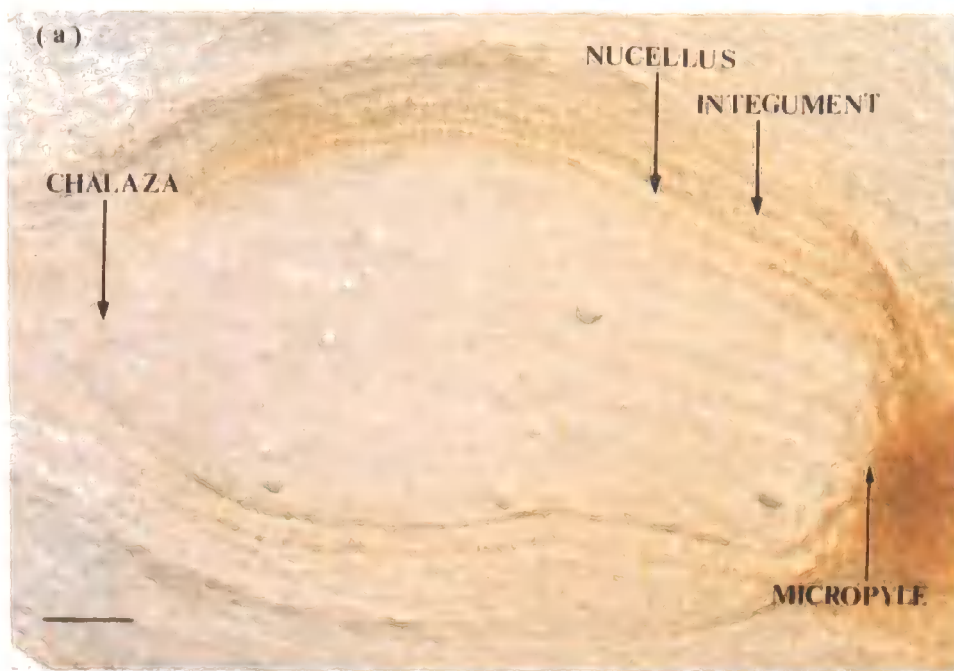
### 3.3 Manipulation of ovaries/ovules and clearance of ESs:

Both the clearing and the enzymic maceration techniques proved effective in revealing ESs of *I. glandulifera* as indicated in Figures (24) and (25) respectively. The embryo is not clearly visible by these methods (Figure 24a), 7 days after pollination *in vitro*.

Isolated embryo sacs of *I. glandulifera* were tested by the FDA test and they showed no sign of viability as indicated by their negative fluorochromatic reaction.

The enzymic maceration technique revealed isolated ESs (Figure 25) with intact ES walls, particularly at the chalazal and micropylar zones. This finding agrees with that of Zhou and Yang (1985) whose results provided evidence of the presence of cutin in the snapdragon ES wall, which may be the reason why ovular tissues macerated enzymically remain intact, the cutin being resistant to the enzyme action.

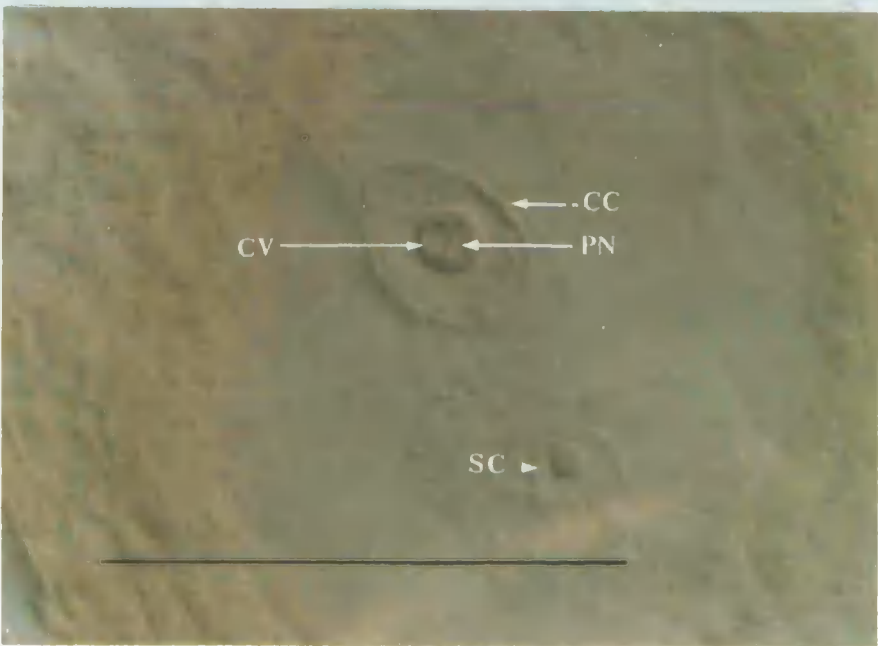
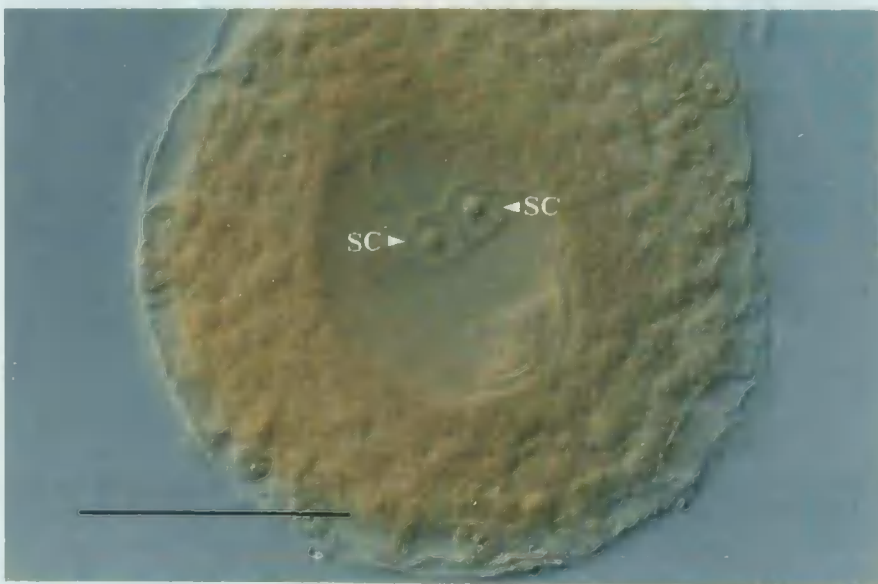
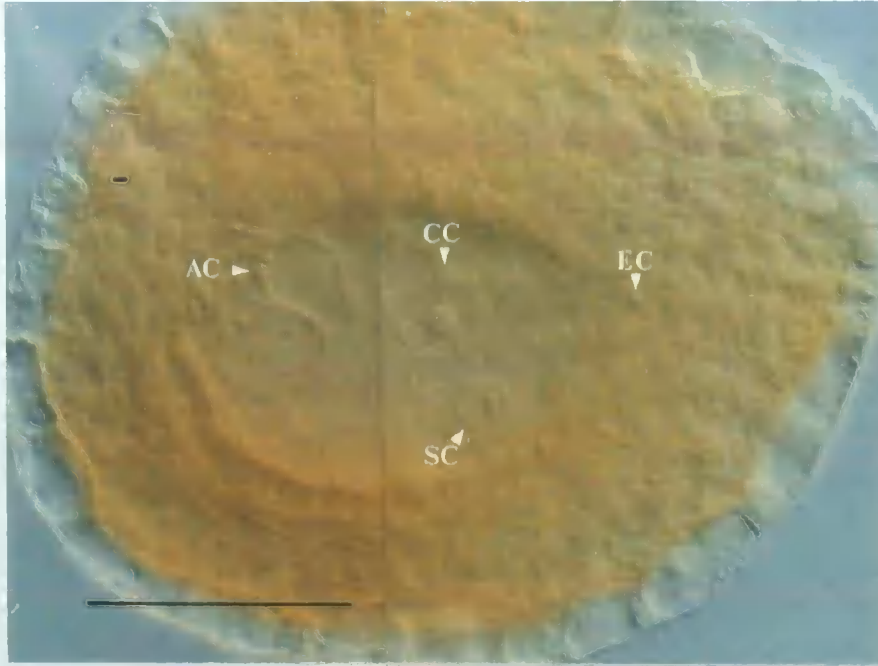
On the other hand, the response of *N. tabacum* to the clearing technique was much better than that of *I. glandulifera* in terms of the clarity of the ES elements (Figure 26). DIC photographs clearly showed ES elements such as the egg cell, the central cell with polar nuclei and central vacuole, the synergids, and antipodal cells. The structure of ES elements observed in this study agrees with the results obtained by Hu Shi-yi *et al.* (1985) who used a prolonged 8 h enzymic maceration technique. The egg cell is smaller than that of the synergid, and the antipodal cells were smaller than the egg. But the central cell was the largest and possessed two polar nuclei and a large central vacuole.



**Figure(24):** Clearance of embryo sacs from *Impatiens glandulifera* ovules (a) at anther opening and (b) 7 days post-pollinating in vivo, using fixing and clearing solutions as described in Protocol 2.3.12. (Bars = 50  $\mu$ m).



**Figure(25):** Enzymic maceration of *Impatiens glandulifera* embryo sacs after incubation of ovules in enzymic solution (Protocol 2.3.13) for 4 h. (Bars = 50  $\mu$ m).



**Figure(26):** Clearance of embryo sacs from *Nicotiana tabacum* ovules at anther opening, using fixing and clearing solutions as described in Protocol 2.3.12. Embryo sac elements shown are the egg cell (EC), central cell (CC) with two polar nuclei (PN) and a central vacuole (CV), synergid cells (SC), and two antipodal cells (AC). (BARS = 50um)

### 3.4 *In vitro* ovule culture:

Of the 15 culture media described in Table (12), *Nicotiana tabacum* ovules grew better in MS10, MS11 and MS12. Ovules growth was normal and no or less damage was caused on the developing ovules compared to other media. As shown in Figure (28), the damage caused by the culture media could clearly be seen in ovules culture in MS10 and MS11, compared to MS12, in which no damage to the developing ovules could be seen. This, however, indicated that MS12 is most appropriate for *N. tabacum* ovule culture *in vitro*. It appeared that *N. tabacum* ovules grew normally in MS12, however, no germination resulted.

When whole ovaries from *I. glandulifera* were cultured in the media described in protocol (2.1.3.1), ovary development *in vitro* was normal for the first 3 weeks (Figure 30b) and the ovaries either shrank or darkened in colour 1 week later, and no seed setting resulted in all cases.

### 3.5 *In vitro* fertilisation:

The result for *in vitro* pollination of *I. glandulifera* ovaries cultured *in vitro* is shown in Figure (27a, 27 b)). The pollen tube can be seen entering the ovule. When *B. napus* ovaries cultured *in vitro* were pollinated by pollen plasmolysed in 0.65 M mannitol in the presence of 1 mg ml<sup>-1</sup> LY-CH and deplasmolysed in 0.30 M manitol, the fluorescent probe appear in the pollen tube entering the ovule (Figure 27c).

When *N. tabacum* ovules were cultured in Nitsch and Nitsch (1969) medium, and pollination was carried by dusting sterilised pollen on cultured ovules, fertilisation took place and embryo germination was obtained as evident from the rooting and shooting shown in Figure (29). However, no embryo germination was shown by the control 'unpollinated ovaries'. Therefore, *in vitro* pollination of cultured *N. tabacum* ovules is quite promising compared to similar pollination attempts carried with *I.*

*glandulifera* and *B. napus*, in which pollen tubes entered the ovules, as assessed by protocol (2.2.8), no embryo germination could be obtained using the Nitsch and Nitsch (1969) medium.



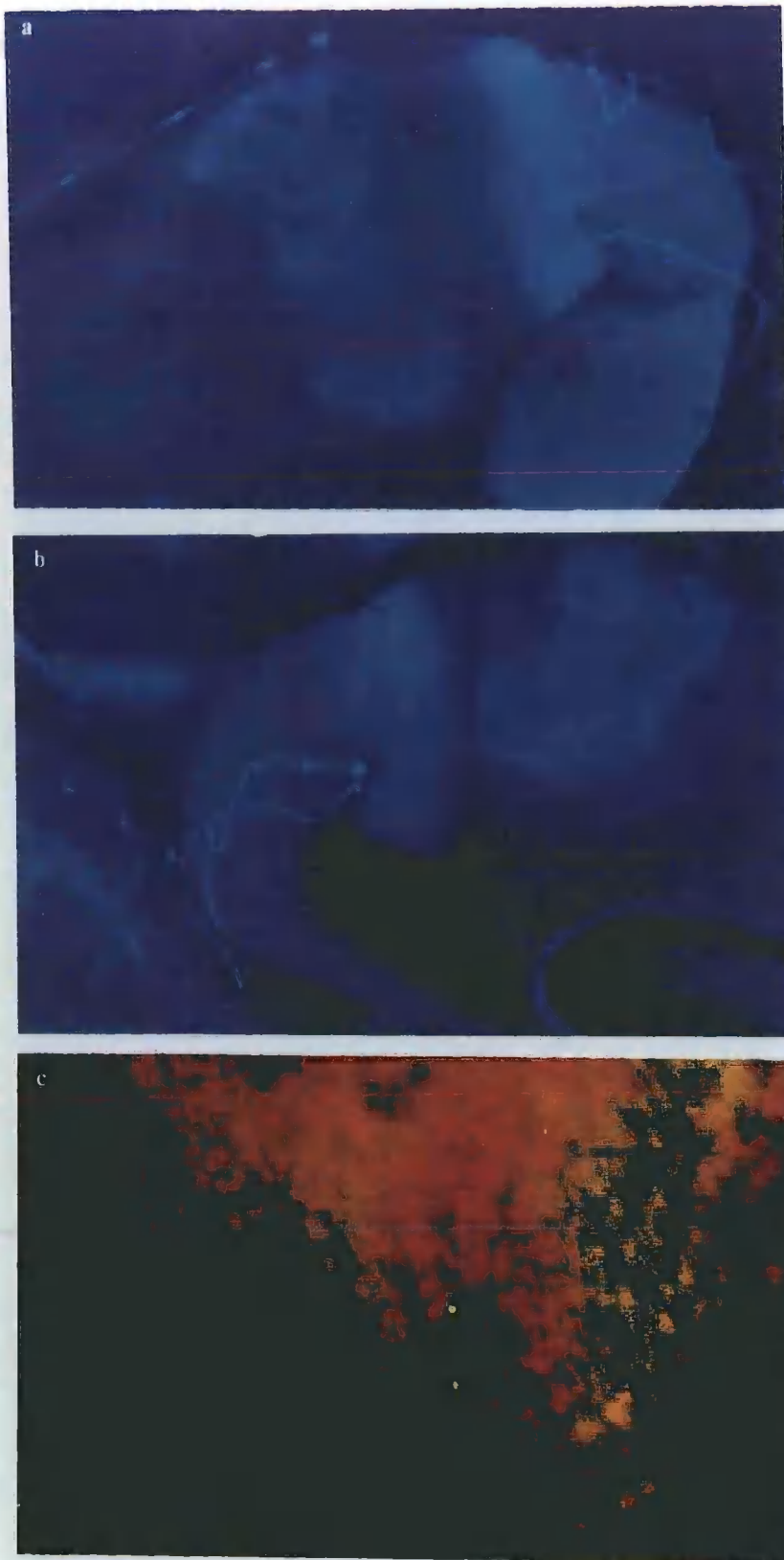
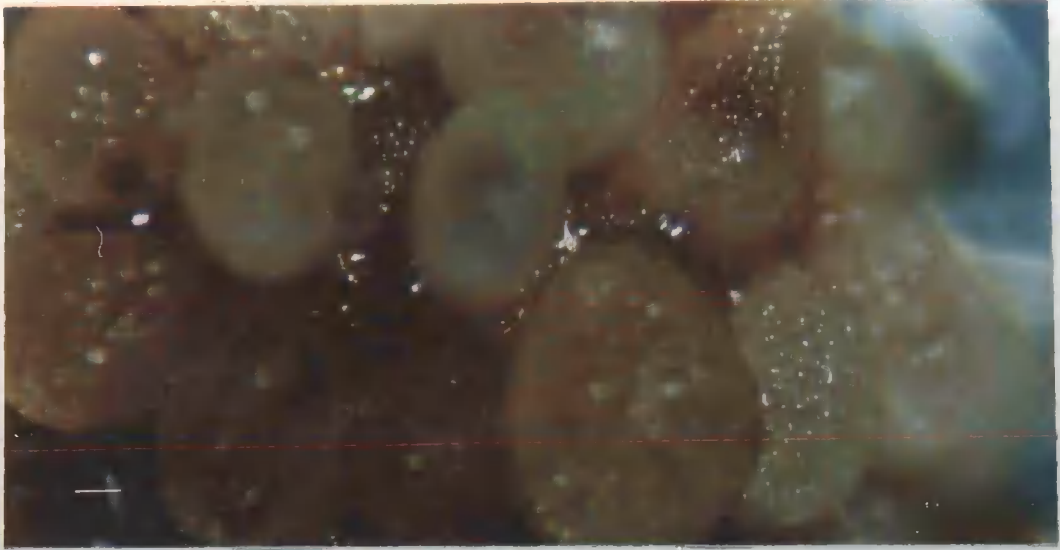


Figure (27): (a) One pollen tube and (b) two pollen tubes of *I. glandulifera* penetrating into the ovule 3 days after stigmal pollination *in vitro*. (c) *Brassica napus* pollen tubes loaded with LY-CH penetrating into the ovule after stigmal pollination with pollen plasmolysed and deplasmolysed in the presence of the probe.



**Figure (28):** *Nicotiana tabacum* ovules 2 weeks after *in vitro* culture in MS-based media: (a) MS10, (b) MS11 and (c) MS12 as described in Table (12). (Bars = 50 $\mu$ m).





**Figure (29)** *In vitro* produced *N. tabacum* plants 7 weeks after *in vitro* pollination of ovaries cultured in Nitsch and Nitsch (1969) medium.

### 3.6 Production of a genetically uniform line:

Attempts were made to micropropagate *Impatiens glandulifera*, *Brassica napus* and *Nicotiana tabacum*. The results are shown in Figures (30, 31).

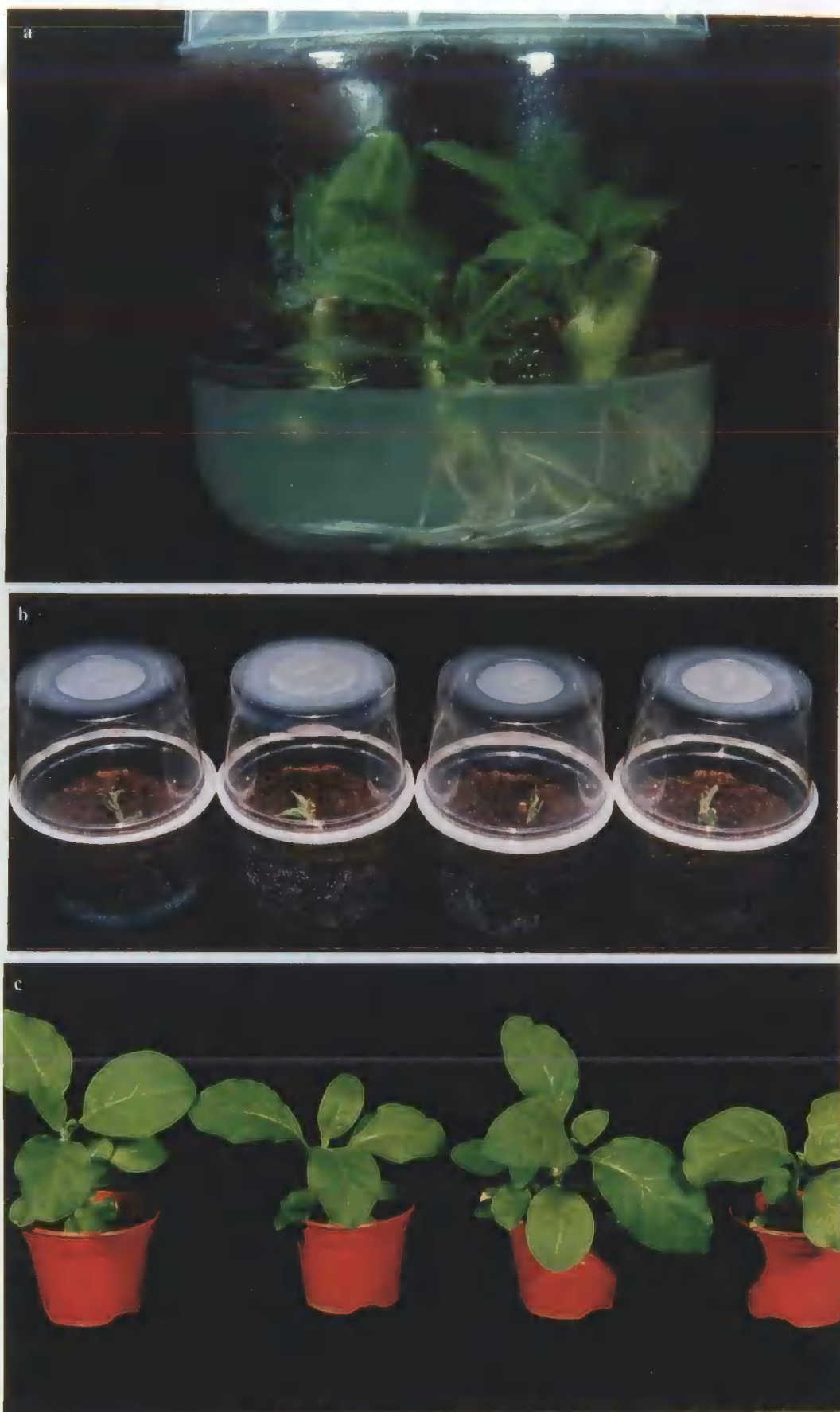
As shown in Figure (30a), single nodes from *I. glandulifera* cultured in MS-based media (see 2.1.3.1) showed abundant chlorophyllous callus in MSO medium to which no hormones were added, compared to MS1, MS2 and MS3. However when small pieces of the callus were subcultured in solidified (0.6% (w/v) agar) and liquid (under agitation) MSO (no growth hormones added), and in solidified (0.6% (w/v) agar) and liquid MS1, MS2 and MS3 to which growth hormones were supplemented (see protocol 2.1.3.1), the callus remained undifferentiated.

Attempts to produce a genetically uniform line in *B. napus* were more promising compared to those in *I. glandulifera*. Whole pistils with pedicels from *B. napus* cultured in Nitsch and Nitsch (1969) medium (see 2.1.3.2) initiated roots and shoots 3 weeks after culture as shown in Figure (30c). When the single node culture technique was applied using nodes from the same plant, no regeneration resulted. This indicated that the potential for micropropagating *B. napus in vitro* is more promising, however, the ability to regenerate varied from one organ to another in the same plant material under similar culturing conditions. This is clear from the response to culture of whole ovaries including pedicels which regenerated, compare to cultures of single nodes from the same plant which did not regenerate.

When the single node culture technique was applied to *N. tabacum* using Nitsch and Nitsch (1969) medium, nodes produced roots 10 days after incubation (Figure 31a). The resulting plantlets responded well to transplanting under humid conditions (Figure 31b) and healthy *N. tabacum* plants were obtained (Figure 31c). Therefore, *N. tabacum* showed a greater ability to regenerate *in vitro* compared to *I. glandulifera* and *B. napus*.



**Figure (30):** Production of a genetically uniform line in *I. glandulifera* and *B. napus*. (a) *I. glandulifera* node setting callus 3 weeks after culture in MSO medium. (b) *I. glandulifera* ovary 3 weeks after culture in MSO. (c) *B. napus* whole pistil and pedicel initiating roots and shoots 3 weeks after culture in Nitsch and Nitsch medium.



**Figure (31): Production of a genetically uniform line in *N. tabacum*. (a) rooting and shooting of nodes 10 days after culture in Nitsch and Nitsch medium. (b) transplanting of plantlets to composte under humid conditions. (c) plants successfully coping with *in vivo* conditions.**

## CHAPTER FOUR

### DISCUSSION

#### ● **Pollen culture *in vitro*:**

#### ◆ **Pollen Growth *in vivo*:**

Although several attempts have been made to formulate an optimal culture medium to improve pollen tube growth, no method has yet resulted in pollen germination and tube length as good as that obtained in nature (Vasil, 1960). Most of the nutrient media developed so far are mainly comprised of sucrose, boric acid, and other nutrients (e.g. Ca, K, Mg). The role of these nutrient compounds is discussed in the Introduction chapter. Only in a few cases (cited in Vasil, 1987 pp 158) does the length of pollen tubes *in vitro* equal that in nature. In pollen grains of *Tradescantia paludosa*, for instance, pollen tubes even grow at about the same rate in liquid suspension cultures as in the style (Mascarenhas, 1966).

#### ◆ **Role of Sucrose:**

Pollen tube length in *I. glandulifera* decreased as sucrose concentration was increased from 5% to 20% w/v. This finding does not agree with Vasil's (1960, 1958a, 1958b) observation in which he described a positive correlation between the concentration of sucrose, and the percentage of germination and tube length. It seems likely that the variation in response may be species-specific.



Nevertheless the results obtained with *I. glandulifera* reveal that the role of sucrose is two fold: osmotic regulation and nutrition. This is because longer pollen tubes were obtained at lower concentration of sucrose (5% (w/v)) compared to higher concentrations (20% (w/v)). The 5% (w/v) sucrose concentration may be considered saturation with respect to nutrition. This agrees with the suggestion of Brink (1924) who suggested that sugars may regulate the osmotic pressure as well as supply nutrition to the growing tubes. Visser (1955) considered the sole function of the sugar is to regulate the osmotic pressure of the solution without contributing to the nutrition of the pollen tube. The theory of endogenous nutrition of the pollen has been disapproved by O'Kelley (1955, 1957) who used C<sup>14</sup>-labelled sugars, and has proved conclusively that pollen tubes do utilise the sugars present in the nutrient solution.

◆ **Role of Boron:**

Longer pollen tubes were obtained from *I. glandulifera* pollen when sucrose and boric acid were combined in one medium. This finding is in line with those of Gauch and Dugger (1953) who postulated that borate ions react with sugar molecules to form an ionisable sugar-borate complex which moves through the cellular membranes more readily than non-borated and non-ionised sugar molecules. Gauch and Dugger (1953) stated that boron appears to be the "dominant factor in the movement of sucrose (or its hydrolytic products) from cell to cell in the plant).

The fact that pollen from *I. glandulifera* germinated and tubes grow in boric acid (from 0.005% 0.02% w/v)) alone, while pollen burst in water and no germination resulted, may suggest that boron (at

0.01% w/v) could be preventing pollen from bursting i.e. has some effect on water relations. The germination of pollen from *I. glandulifera* in boric acid (without sucrose) may be taken as evidence that boron could have combined with the endogenous sugars and led to pollen germination and tube growth. Concentrations of boron higher than 0.02% (w/v) were reported by Vasil (1960) as toxic, and can adversely affect the percentage of germination as well as the tube length. However, plants differ in their requirements of boron according to their natural level of boron.

◆ **Role of Minerals:**

Other chemical substances reported to improve pollen germination and pollen tube growth, as mentioned earlier, include calcium, potassium, magnesium and others. However, the effect of sucrose and boric acid far surpasses the effect of any other chemical substance or hormone so far used in pollen culture.

As evident from the increase in tube length of pollen from *I. glandulifera* cultured in a sucrose/boric acid medium supplemented with Ca, Mg, and K, compared to the control treatment (no minerals added), it can be observed that these minerals do promote pollen tube growth *in vitro*. The role of minerals can be clearly defined from the findings of Brewbaker and Kwack (1963) who studied the pollen population effect in small and large pollen populations. They observed that a population effect occurs whenever pollen grains are grown *in vitro*, i.e. single pollen grains don't germinate *in vitro*, but populations of minimum numbers will. Pollen itself carries essential nutrients but not in high enough concentrations for growth, in a single pollen grain. Brewbaker and Kwack (1963) concluded that this factor is shown to be due to the calcium ion. The action was confirmed in 86 species. Other

ions ( $K^+$ ,  $Mg^+$ ,  $Na^+$ ) serve in supporting roles to the uptake or binding of calcium.

As indicated earlier, pollen growth rate in *I. glandulifera* showed a decline after 2 h of incubation of pollen *in vitro*. This suggests that either the osmotic concentration and nutritional value of the basal medium deteriorated with time or a more complex medium supplemented by other nutrients is needed. This is actually the case *in vivo* where pollen tube growth is supplemented by metabolites taken up from the stigma and style during penetration towards the ES. Pollen tubes growing *in vivo*, however, control to some extent the nature of medium through which they grow. They solubilise the pectic components of the style secretions by the enzymes they emit from their tips (Loewus and Labarca, 1973) which releases sugars that contribute to the osmoticum, and moderate the passage of water into the tube.

Pollen from *I. glandulifera* readily germinates in a simple sucrose/boric acid medium. The pollen as shed revealed immediate capacity for germination. However, the variability in the results obtained might mainly be attributed to the variations in the water potential of the pollen which, as stated by Heslop-Harrison (1987, pp 13), is governed by the solutes on or in the pollen grain; the matric potential attributable to the colloids of the cytoplasm; and the wall pressure. Non-uniformity with the culture of pollen has also been reported even with pollen from the same anther which suggests that genetically uniform material, assessed under uniform physical and chemical conditions is needed to eliminate such variability. Meiosis in the anther of an outbreeding species like *I. glandulifera* implies that



gene segregation and chiasmata formation will produce populations of pollen grains which are genetically highly heterogeneous.

Such nonuniformity with the culture of pollen from the same anther may also be attributed to an environmental component according to the position of pollen within the anther where a gradient of nutrients exists. Variation in pollen tube growth rate has been seen in other species (Cruzan, 1990). Differences in pollen tube growth rates are consistent across maternal genotypes (Snow and Spira, 1991).

◆ **Effect of temperature on pollen growth *in vitro*:**

In most plant species maximum germination and tube length *in vitro* are obtained between 20 °C and 30 °C; however, temperatures higher than that seem to retard the growth of pollen tubes, with a few exceptions (Hirose, 1957, cited in Vasil, 1987) where optimum germination was reported at 35-40 °C.

Temperature was reported to increase pollen tube diameter (Smith, 1942). However, pollen cultured at temperatures higher than 35 °C were reported to show marked swelling of pollen tube tips, branching, and bursting (Vasil and Bose, 1959). The effect of temperature on *I. glandulifera* pollen growth revealed high pollen bursting at temperatures higher than room temperatures, which in turn could lead to subsequent bursting after incubation due to pressure exerted by water uptake.

Incubation temperatures of up to 45-60 °C were reported by Rao *et al.* (1992) to have no effect on the extent of seed set in *Brassica*, compared to that from fresh pollen. The present study has revealed an interesting finding in which pollen from *I. glandulifera* have germinated at lower temperatures (8 °C and 4 °C) which will have an

ecological implication as the pollen tube will continue to grow day and night.

It was reported by Niimi and Shiokawa (1992) in their study on the storage of *Lilium* pollen, that stored pollen grains (4 °C for 9-12 months) may have the *in vitro* germination capacity, but only those from a few species and cultivars have the ability to produce seed. On their study of the differential cold sensitivity of pollen grain germination in two *Prunus* species, Weinbaum et al. (1984) observed some pollen tube elongation at 4 °C. The adaptations of pollen from these species, and that of *I. glandulifera* shown in the present study, to low temperature conditions, may be associated with their adaptation to late-blooming dates, which requires pollen germination and tube growth to proceed at low temperatures.

◆ **Effect of humidity on pollen growth *in vitro*:**

As shown earlier in Figure (8), there was a gradual increase in pollen tube length as relative humidity was increased.

For pollen germination Heslop-Harrison (1987) considered that pollen preconditioning in a humid atmosphere gives time for the reorganisation of the cell membrane in the partly dehydrated grain.

The mature pollen is considered by Hoekstra and Bruinsma (1980) as "inactive metabolically", with a porous and ineffective plasma membrane at the time of dispersal (Heslop-Harrison, 1979). Therefore, the hydration of pollen leads to restoration of membrane integrity and considered an essential prerequisite for successful pollen tube development.

It is not clear why variation in atmospheric humidity above the germination medium should affect pollen tube length. One possibility is

that the physical relationship between atmospheric humidity and the liquid medium regulates evaporation or water uptake from the atmosphere by the medium, creating continuous concentration gradients within the germination medium which enhance pollen tube growth. Perhaps gradients of metabolite concentration are important in pollen tube elongation. It is tempting to speculate that such gradients may have a role in directing pollen tube growth *in vivo*, and that the *in vitro* system employed here somehow mimicked this natural system.

Humidity plays an important role in the way it regulates molarity and provides for optimum osmotic values in experiments dealing with fertilising ovules *in vitro*. Manipulation of atmospheric conditions above the culture medium may also be important in this instance.

- ◆ **Solid Versus Liquid Media:**

The variation in pollen growth rate in liquid and solidified PCM can be attributed to the fact that water flux is faster in liquid PCM, and so nutrient uptake is more rapid. However, it was reported that pollen tube growth *in vitro* continues when the medium shows a gradual decrease of molarity (Van Aelst, 1983) as would be the case with liquid PCM, which could be attributed to the effect of varying humidity in the air space above the medium, controlling the rate of evaporation of the aqueous solvent. Therefore it seems likely that the difficulty in bridging the gap between *in vitro* and *in vivo* pollen tube growth could be overcome by addressing factors related to the forces concerned in water passage, and the hydrostatic pressure that will be built up in the grain.

② **Assessment of pollen viability at different temperature and humidity conditions:**

◆ **The Fluorochromatic Reaction (FCR) Test:**

The most widely used method for assessing pollen viability is based on the FCR Test, which essentially demonstrates the activity of esterases and, more importantly, the state and permeability of the plasmalemma (Heslop-Harrison and Heslop-Harrison, 1970; Rao *et al.*, 1992). The present study shows that preincubation of pollen at low temperatures ranging from 4 °C to RT does not affect the viability of pollen of *I. glandulifera* in terms of the *in vitro* germination test. The FCR Test resulted in lower fluorescence intensity at low incubation temperatures (4-8°C) and a higher intensity of fluorescence was obtained after incubation under RT for 24 h. Pollen preincubated at 60 °C for 24 h was distinctly FCR negative and failed to germinate. However, it was observed in this study that some highly fluorescing pollen was unable to form pollen tubes, while lightly fluorescing pollen did, an indicative that the FCR test shows only an approximate estimate of pollen germinability. Pollen germination percentages lower than FCR values were reported by La Porta and Roselli (1991). This could indicate that the fluorochromatic reaction as a histochemical method, which depends on the presence of active esterase in the pollen grain, together with the integrity of the plasmalemma, does not precisely reflect the actual viability status of pollen grains.

In the light of the FCR results explained earlier, and in the view of Matthys-Rochon *et al.* (1987), the FCR test introduced by Heslop-Harrison (1970) has to be reexamined. In the study by Matthys-Rochon *et al.* (1987) they noticed both for fresh and stored pollen some FCR

negative pollen grains with FCR positive sperm cells. This might be due to pollen grains with defective membranes and sperm nuclei with intact ones.

The fact that fluorescence intensity increased with the increase in incubation temperature compared to the response showed by pollen as shed, could be taken as an evidence that the response of pollen to the FCR test is greatly affected by factors which affect pollen immediately after shedding, i.e. dehydration of the pollen grain after its release from the anther, for instance, may cause domains of the vegetative cell plasma membrane to enter an unstable gel/liquid crystal state (Kerhaos *et al.*, 1987).

From the nonsignificant difference in response of fresh pollen from *I. glandulifera*, *N. tabacum* and *B. napus* to the FCR Test shown in the Results Chapter (Figure 9), one might ask: *why doesn't the FCR Test vary between species?* This could be taken as an indication of the importance of the physiological dimensions of pollen (enzymic activity) compared to the physical ones. However, though it was beyond the scope of this study to assess the enzymic status of pollen, it would be necessary to assess these, before a concrete conclusion is drawn about their significance.

◆ **The CFW Test:**

To explain how the CFW test works, one must refer to the study by Fischer *et al.* (1985) in which they used CFW to distinguish between living and dead cells from a variety of animal and plant species. In their study, they concluded that non-viable cells showed a lightly stained cytoplasm and brightly stained nuclei as a consequence of CFW penetration through a disrupted plasmalemma or cell

membrane. Plasmalemma disruption (CFW penetration) indicates inviability.

In the present study, the response of pollen to staining by CFW showed variation in fluorescence intensity due to variation in incubation temperatures. Higher fluorescence intensity was revealed by pollen incubated at 4 °C compared to higher temperatures, showing a negative correlation as incubation temperature increased to 60 °C. One might ask: *why did the effect of CFW on pollen vary with temperature?*. Of course, CFW binds to cellulose and chitin (Hayashibe and Katohda, 1973), callose and carboxylated polysaccharides (Hughes and McCully, 1975) and a variety of other  $\beta$ -linked polymers (Maeda and Ishida, 1967). It can be argued that temperature might be affecting the biochemical nature of either of these cell constituents in such a way to affect their stainability by CFW as expressed by fluorescence intensity.

Pollen which did not germinate, and therefore was assumed to be non-viable, were observed to show fluorescing organelles due to CFW penetration of the disrupted plasmalemma or cell membrane; a clear distinction between germinable and non-germinable pollen.

◆ **Factors Affecting Pollen Viability:**

From the results explained earlier with respect to both the FCR and the CFW tests, it seems additional factors have to be considered in order to reach an effective technique for evaluating pollen viability, since it does not follow that a living male gametophyte will necessarily be able to form a pollen tube and effect fertilisation. The interaction between the physical, and physiological dimensions of pollen with environmental conditions makes it even more complicated to define the effect of each of these factors on pollen viability. Earlier investigations

by Shivanna et al. (1990) show clearly that high RH and temperature stress affect pollen vigour before they affect pollen viability or germinability. The vigour, as measured by speed of growth and ultimate length of pollen tubes, deteriorates before the ability to germinate declines. In another study by Shivanna and Johri (1984) and Kumar et al. (1988) they reported the loss of vigour without the loss of germinability. The effect of high RH and high temperature stresses on viability of *N. tabacum* was reported by Shivanna et al. (1991) as nonsignificant in terms of pollen viability on the basis of the fluorochromatic reaction (FCR) test, nor did it affect pollen vigour, on the basis of the time taken for in vitro germination. However, pollen vigour was markedly affected when both stresses were given together.

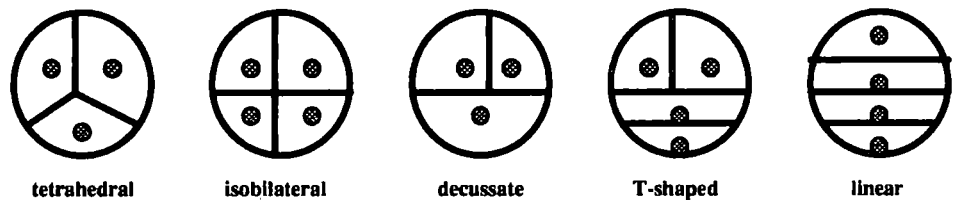
The behaviour and survival of pollen are influenced by both environment and genotype. Pollen viability varies with the nutrition of the parent plant. It also requires pre-conditioning and post-maturation development. Some viability testing methods may give an unrealistic estimate of quality (Heslop-Harrison, 1992).

### ③ **Pollen developmental state and monitoring of pollen nuclei:**

The use of the Feulgen staining technique was an effective tool in defining the various developmental stages in *I. glandulifera* pollen, particularly late meiosis. This technique, in conjunction with DAPI, acridine orange and aniline blue has significantly contributed to the understanding of the various developmental stages of pollen and identification of the structure and behaviour of vegetative and generative nuclei, which is actually an essential tool that helps in understanding pollen biology, assisting in manipulation of pollen both

*in vivo* and *in vitro* at any particular growth stage. Some of these parameters will be discussed. (See also Chapter Two: 2.4).

Cell division in plants occurs in two basic steps: mitosis, the division of the nucleus, and cytokinesis, the division of the cytoplasm. Microspores are usually arranged in tetrahedral or isobilateral fashion, but there are exceptions. A decussate arrangement has been recorded in *Magnolia* (Farr, 1918), *Atriplex* (Billings, 1934), *Cornus* (D'Amato, 1946), and many other plants. Linear tetrads in which the mother cells divide transversely has been reported in some genera of Asclepiadaceae (Gager, 1902) and in the genus *Halophila* of the Hydrocharitaceae (Kausik and Rao, 1942). T-shaped tetrads also occur sometimes, as in *Aristolochia* (Samuelsson, 1914) and *Butomopsis* (Johri, 1936). The diagram below shows the different types of microspore tetrads:

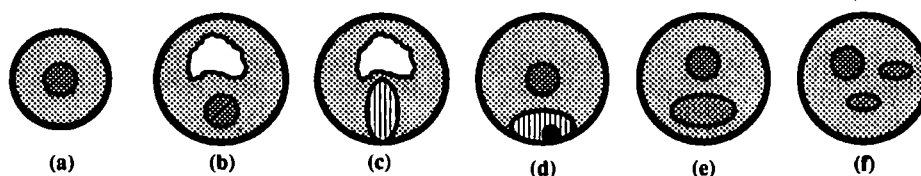


The type of microspore tetrad in *I. glandulifera* is an isobilateral arrangement in which the microspore mother cell divides to form four microspores. In some plants (e.g. *Drimys*, *Anona*, *Drosera*, *Elodea*, *Typha*, *Furcraea* and several others) it was reported that microspores adhere in tetrads to form the so-called "compound" pollen grains (Wodehouse, 1936; Erdtman, 1943, 1945).

The development of the male gametophyte in angiosperms can be sketched in the following illustration (modified from Maheshwari, 1949): (a) newly formed microspore. (b) vacuolation and wallward position of nucleus. (c) nucleus dividing. (d) vegetative and generative



cells. (e) generative cell free in cytoplasm. (f) division of generative cell.



It has been reported by Wulff (1934) and Heitz and Résende (1936) that the pollen nucleus in *Impatiens* may show a pro-metaphase stage and that the division of the generative cell occurs by a constriction (Raghavan et al., 1939). The present study reveals that division of pollen nucleus to vegetative and generative nuclei takes place immediately after the tetrads break up to single microspores.

Details of the position and functional importance of the vegetative nucleus vary depending on whether it is always in the distal end of the pollen tube or behind the male gametes. Its importance in directing the growth of the pollen tube has been questioned by Poddubnaji-Arnoldi (1936), where he reported growth of branched tubes, as has been observed in this study with *I. glandulifera*, although only one of them contains the nucleus. Early degradation of the vegetative nucleus has been reported (Heweitt, 1939).

#### ④ **Plasmolysis/deplasmolysis of pollen, *in vitro* pollination and identification of pollen tubes:**

The use of decolourised aniline blue has proved to be an effective technique that permits rapid localisation and visualisation of pollen tubes. This study has revealed that the first callose plug is not necessarily formed at a fixed distance from the tip of the pollen tube as stated by Vasil (1987). However, even the shape of callose plug varies

in pollen tubes growing under similar growth conditions *in vivo* and *in vitro* (see Figure 17 and 18).

The role of callose plugs is essential in limiting the pollen tube cytoplasm, its streaming, and confining nuclei to the terminal part of the tube. They also help maintain the turgor pressure in the terminal part of the tube (Vasil, 1987). The variation in density and distribution of callose plugs along pollen tubes reported in this study might reflect the way pollen responds to the surrounding growth conditions. However, the use of callose plugs as an indicator of relative pollen tube growth rate (Snow and Spira, 1991) could only apply to species in which callose plugs are deposited at regular intervals, as shown in *N. tabacum* earlier in this study.

The multiple pollen tubes shown by germinating *I. glandulifera* pollen (Figure 17e-g), though not a commonly observed feature of the species, might be considered an interesting feature *in vitro*. There is no evidence that it occurs *in vivo*. It is a valuable tool for looking at relationship between the pollen tube and the vegetative nucleus. However, the fate and role of pollen nuclei in multiple pollen tubes is an interesting aspect for future research programmes to consider.

#### ⑤ **Loading of fluorochromes into pollen by Plasmolysis/endocytosis:**

Endocytosis is defined by Oparka *et al.* (1991) as "the mechanisms by which eukaryotic cells ingest selected regions of their plasma membrane" resulting in the formation of endocytic vesicles which consist of membrane components (protein and lipid) as well as material which binds to the extracytoplasmic face of the plasma membrane, and solutes dispersed in the fluid phase of the extracellular

medium. Oparka et al. (1991) discussed in details receptor-mediated and fluid-phase endocytosis. However, they did not rule the occurrence fluid-phase endocytosis in plant cells under normal osmotic conditions. Many of the arguments against endocytosis were based on the condition that coated vesicle-mediated endocytosis may not be possible at turgor pressures greater than 1 bar (Grandmann and Robinson, 1989); however, as mentioned earlier, there can be rapid changes and huge fluctuations and variability in turgor pressure in plants.

◆ **Loading of LY-CH:**

Since its introduction in 1978, LY-CH has been used with considerable success as an intracellular marker in a wide variety of biological systems. It was reported for the first time by Hillmer *et al.* (1989) that plant cells can take up the fluorescent dye and deposit it in the vacuole.

The results shown in this study provide new evidence of uptake of LY-CH by pollen after plasmolysis and deplasmolysis. Microtome sections (4  $\mu\text{m}$  thick) have clearly shown the accumulation of LY-CH around the pollen nuclei, which agrees with the finding by Oparka et al. in which they reported vesicles trapped in the thin layer of the cytoplasm surrounding the nucleus during the rapid plasmolysis of onion epidermal cells. As the probe does not appear to diffuse across the plasma membrane (Miller, et al. 1983), and also on the basis of similarity of uptake of LY-CH with other known endocytic markers (Buckmaster *et al.*, 1987), the most likely mechanism by which this dye was taken up by pollen was through endocytosis.

◆ **Loading of Fluorescein:**

Fluorescein is reported to be partially undissociated in the region of pH 2-6, thereby allowing diffusion of the neutral form of the molecule across the membrane (Oparka *et al.* (1991)). Its uptake by pollen, as evident from formation of fluorescent vesicles, can only be attributed to endocytosis as it was taken up at pH 7.7. The loading of fluorescein into pollen and the formation of fluorescent vesicles supports the evidence by Oparka *et al.* (1991) that fluid-phase endocytosis can occur in plant cells under normal osmotic conditions. However, by comparing the mobility of fluorescein into pollen shown in Figures (15c) and (23), it can be observed that, in Figure (23) the probe did not, in a number of pollen enter the vacuole, which appeared as a dark spot; compared to the mobility of fluorescein shown in Figure (15c). This could be attributed to the difference in pathway employed to load the probe, as in the first case pollen were directly immersed in the probe, while in the second case the probe was loaded via the pedicel in which case the concentration and pH of the probe might have changed on the route via the pedicel up to the anther.

The fact that no endocytic vesicles were observed (using Nomarski DIC), when pollen from *I. glandulifera* were immersed in distilled water, in comparison with the formation of endocytic vesicles and uptake of LY-CH by plasmolysis and deplasmolysis, at the times no direct penetration of the plasma membrane was observed (where no plasmolysis was employed), may be taken as evidence of the induction of endocytic vesicles osmotically. By contrast, the formation of such vesicles in the case of fluorescein where no plasmolysis and deplasmolysis was employed, might be taken as evidence of the formation of endocytic vesicles by fluid phase endocytosis, however, it

remains to be tested whether clathrin is somehow involved in the process as suggested by Oparka *et al.* (1991).

The key significance of this study is the use of fluorochromes in combination with endocytosis. Plasmolysis/deplasmolysis has been used as a means of transporting molecules, in the hope that they would at least survive to be incorporated into the zygote. The fate of these membrane-bound vesicles could possibly be as follows:

- (1) They may fuse together.
- (2) They may fuse with internal membrane (the endocytic reticulum).
- (3) They may be transported across the cell.
- (4) The membrane may break down and discharge probe into cells.

Of all the vesicle trafficking systems, the process of receptor-mediated endocytosis is considered as the best characterised (Hawes *et al.*, 1991). On their study on protoplasts of soybean, Fowke *et al.* (1991) using cationised ferritin (CF), discovered that CF is internalised by coated pits to coated vesicles and subsequently delivered to cytoplasmic organelles in soybean protoplasts. They concluded that the endocytic pathway involves endocytosis by coated pits, delivery to coated vesicles, smooth vesicles, the partially coated reticulum and Golgi, multivesicular bodies and finally to the cell vacuoles. However, the endocytic pathway of fluorescent vesicles observed here, that resulted from loading of LY-CH by plasmolysis/deplasmolysis, and accumulated around pollen nuclei, is not understood.

⑥

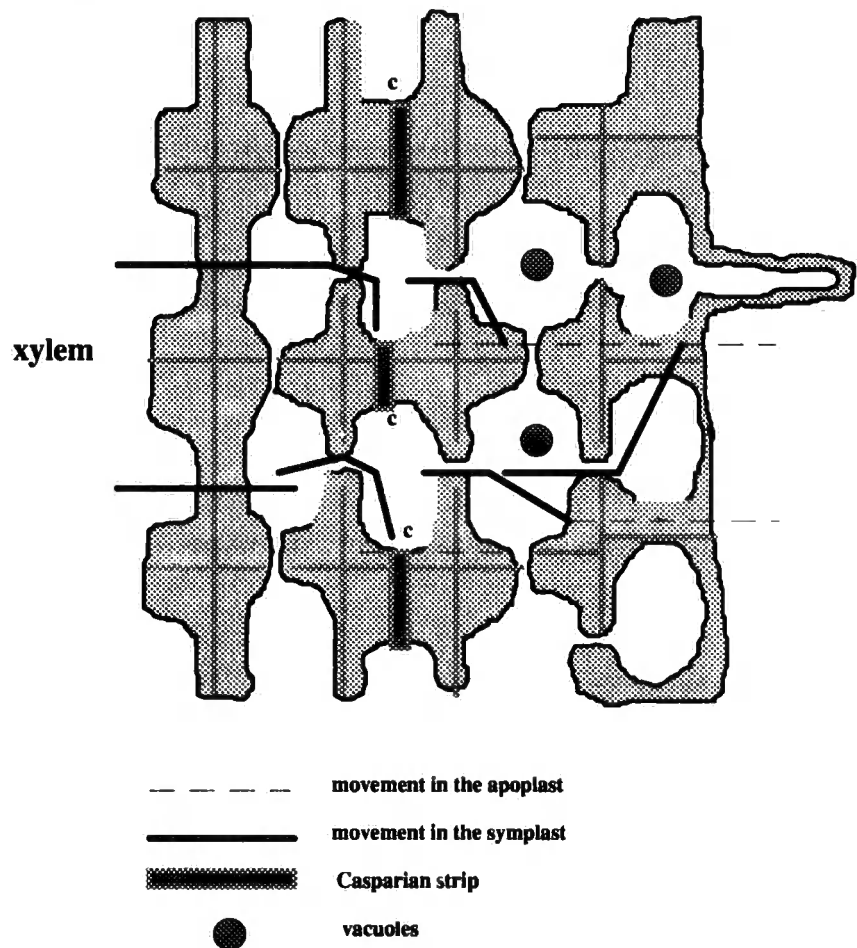
### **Loading of fluorochromes into plant reproductive cells via the vascular system:**

Fluorescent probes are defined as molecules which are "intrinsically fluorescent when viewed under radiation of differing wave lengths", which is the case with LY-CH and fluorescein used in this study. Fluorescent stains, on the other hand, are those "molecules which fluorescence only after binding to a chemical constituent of the cell" (Oparka, 1991) as is the case with CFW.

A number of authors used fluorescent dyes to trace both symplastic (Palmquist, 1939) and apoplastic (reviewed in Canny, 1990) transport pathways within plants. The apoplast is defined as the continuum of non-protoplasmic matter, such as cell walls and intercellular material, throughout a plant. The symplast, on the other hand, is the continuum of cell protoplasts throughout the plant, linked by plasmodesmata which allow the passage of material between cells.

The vascular system is the conducting apparatus of the plant. It consists of a system of discrete strands known as the vascular bundles, each of which consists of both xylem and phloem forming a ring around the central pith. Within the xylem, there are two types of conduits, composed of tracheids or of vessel elements which have thick, lignified secondary walls which permits the movement of large volumes of solution with no cytoplasmic or membrane resistance to overcome. In addition to that, the xylem parenchyma is also involved in water and solutes transport into and out of the conducting cells. The phloem, on the other hand, is composed of sieve elements which occupy some part of the tissue, the remainder being companion cells, parenchyma and fibres.

In a study of solute transport of plants, Delrot (1987) regarded the apoplast and symplast as 'mutually exclusive transport compartments', which implies that a particular solute molecule may move freely in either one or the other. The following simplified diagram (modified from Moorby, 1981, p 89) shows the pathways followed by water and ions as they move across the root to the xylem:



It is necessary, at this stage, to review the characteristics of fluorescent probes used in this experiment and highlight the route of uptake in each case.

◆ **Loading of LY-CH:**

The importance of LY-CH for microinjection studies and also for fluid phase endocytosis studies stems from its unique properties.

These include low toxicity to cells, high quantum yield, stable quantum yield between pH 1-10, high solubility in water, lipid insolubility and high dissociation at physiological pHs (Oparka, 1991).

LY-CH was reported to enter the vacuoles of potato storage parenchyma cells following its introduction into the apoplast (Oparka and Prior, 1988). However, recent studies indicate that the dye is unable to cross the plant cell plasmalemma (Terry and Robards, 1987), so it seems likely that the dye is transported from the apoplast to the vacuoles of the parenchyma by an endocytic mechanism (Oparka *et al.*, 1988).

Experimental results discussed earlier (Chapter 3.2) have shown the uptake of LY-CH via the pedicel through the xylem cells. However, translocation of the probe from the vascular system of the pedicel to routes leading towards floral appendages gives a clear picture of possible interactions among the various parts of the flower. The vascular network between the pedicel and floral appendages, however, conforms to the concept of Steeves and Sussex (1972) that the vascular system in the stem may be described as being largely, if not entirely, leaf-oriented.

The primary purpose of the present study was to investigate the use of pollen for transferring xenobiotics to embryo sacs, but the incidental observations on transport of fluorescent probes via the transpiration stream to the ovules merit some comment.

The differences in the accumulation of LY-CH in the funiculus of *N. tabacum* (Figure 20a) and *I. glandulifera* (Figure 20b) may reveal some useful information with regard to the structural nature of the funicle and its relation to the development of vascular tissues within the ovule wall. The differences might be explained in terms of species-



specific variations in the state of differentiation and/or physiological activity in the xylem and phloem tissues. In some species it is known that the vascular tissue on the ovule wall does not differentiate or become fully functional until after fertilisation. Further investigation of *I. glandulifera* ovule anatomy and physiology is required, to determine the structure/function relationships in this species.

The accumulation of LY-CH in the funicle and outer layers of the ovule wall might also be a result of variations in the permeability of the embryo sac, which may be a function of callose deposition in its wall and the relative impermeability of this substance (Heslop-Harrison 1964, 1966). Rodkiewicz (1970) observed that "it seems possible that the permeability of the cell wall containing callose is less than that of cellulose cell walls".

Clearly, this differential accumulation at the funicle/ovule interface in different species merits further study.

The presence of traces of LY-CH in the style, and particularly in the stigma cells of *N. tabacum*, is particularly interesting with respect to the manipulation of pollen and the loading of xenobiotics via the pedicel. It suggests the possibility of loading small molecules via the apoplastic transport stream that might then be transferred to the pollen symplast via the stigma/pollen interface. Stigmatic pollen germination might then result in pollen tubes that would transport this material to the embryo sac, in the symplastic domain of the maternal plant. If successful, such a route for loading xenobiotics might be particularly valuable, since it would avoid the potentially damaging effects of plasmolysis and deplasmolysis on pollen and would allow almost all xenobiotics loading, pollen germination, pollen tube growth and fertilisation steps to be carried out *in vivo*. There was insufficient

time in the present study to assess the value of this protocol, but it should be investigated in further work.

- ◆ **Loading of CFW:**

CFW is a fluorescent stain which fluoresces only after binding to a cellular chemical constituent of the cell wall. The optical brightener has been used to stain cell walls of higher plants. It binds strongly to cellulose, carboxylated polysaccharides, callose, with perhaps some binding to lignin (Hughes *et al.*, 1975). However, the specificity of binding to chitin was questioned by Johnson *et al.* (1974).

The use of CFW as a marker for movement in the apoplast of plant cells requires some information on the permeability of plant cells. Uptake of CFW via the pedicel of *I. glandulifera* took place apoplastically and the probe was clearly visible in the funiculus and around the embryo sac as shown in Figures 21a and 21b. This finding supports similar research findings in which CFW was used as an apoplastic tracer (Gunning and Hughes, 1976).

- ◆ **Loading of fluorescein:**

Uranin (sodium fluorescein) was described by Tyree and Tammes (1975) as water soluble, moves within the symplast, and can be detected easily with fluorescence microscopy. The probe was first used by Schumacher (1933) who reported that the dye entered the symplast, and could also be accumulated and transported by the phloem. Palmquist (1939) used fluorescein to study phloem transport in potato and kidney bean, and he noticed that the dye did not remain confined to the phloem but frequently leaked to the apoplast, ending up in the transpiration stream (Palmquist, 1938). It was also reported

(Grignon *et al.*, 1989) that fluorescein can leak into the apoplast while being transported by phloem and carried by the water stream towards xylem tissues. They attributed that to the large apolar structure of fluorescein compared with that of 6(5)carboxyfluorescein in which the supplementary acidic group eliminates the permeating effect of the apolar part of the molecules (Weinstein *et al.*, 1977).

The interesting point about fluorescein fed via the apoplast is that it end up in pollen and the embryo sac (Figures 23 and 22), showing that it travels from the apoplast to the symplast at those sites and crosses the boundary between the sporophytic and gametophytic generation, which suggests that it must be entering pollen and the embryo sac via endocytosis, without an intervening plasmolysis step.

Palmquist (1939) detected greenish yellow fluorescence, characteristic of fluorescein, in the phloem of the petioles of bean leaves to which fluorescein had been applied. He further reported that fluorescein can be made to move more rapidly in the xylem than in the phloem. Once fluorescein, or any other solute had entered the xylem, it moves with the transpiration stream. However, the apparent movement in the phloem is merely the result of lateral diffusion.

There are two key factors that seem to have effect on translocation of fluorescein. The first factor, as pointed out by Rhodes (1937), is that fluorescein might be present in the xylem in a non-fluorescent state, since it does not fluoresce strongly in acid solution. This does not seem to apply to the pattern of LY-CH translocation, as the probe is characterised by high dissociation at physiological pHs (Oparka, 1991).

Fluorescent probes, such as uranin, has been used to study the pathway of solutes into the ovule and ES (Mogensen, 1981), in an

attempt to identify which cells are involved in the process of ES nutrition. However, the manner of probe uptake by ovules has greatly been influenced by the type of ovule. Anotropous ovules (*Nicotiana tabacum*), showed uranin uptake in a nondifferential manner; atropous ovules (*Polygonum capitatum*) showed differential uptake into the small group of cells at the micropylar end of the ovule, and thus, near the egg apparatus; and hemianatropous ovules (*Torenia fourneiri*) showed uranin uptake into the egg and synergids (Mogensen, 1981).

It may be emphasised at this stage that the use of the previously mentioned probes could be extremely valuable in defining nutrient transport pathways, especially those concerned with the developing embryo, the knowledge of which is very limited. The nutritive pathway during megasporogenesis and megagametogenesis, however, varies with the ovule developmental state (Willemse, 1992). He stated that the nucellus is in close contact with the placental tissue and the growing cells obtain their nutrition via symplastic and apoplastic transport. However, during the end of meiosis, the hypostasis appears as a local apoplastic barrier of phenol-containing substances, and the nutrient flow is partly changed to a symplastic flow only.

Willemse (1992) outlined exceptional transport routes via the pollen tube, or through haustoria formed by the synergids or other cells of the ES. In the present study, the pollen tube route has proved to be a possible route for loading of fluorochromes (LY-CH) as shown with *B. napus*.

Uptake of fluorescein loaded via the pedicel by *I. glandulifera* pollen reveals an important route via which xenobiotics can be loaded into pollen. This route could be of paramount importance in pollen manipulation *in vitro*.

### **Manipulation of ovaries/ovules and clearance of ESs:**

The ES clearing and enzymic maceration techniques adopted in this study were successful in the clearing of ES elements as well as in the isolating of intact ESs of *I. glandulifera* and *N. tabacum*. However, the embryo sacs isolated by enzymic maceration of *I. glandulifera* ovules were not viable as expressed by their negative fluorochromatic reaction, most probably because of the enzymic degradation of the cellulose in the wall during isolation. The reliability of such techniques often varies from plant to plant according to ovary and ovule size and structural complexity. The fact that the ES is deeply buried in the ovule tissues makes it beyond the reach of biologists, compared with its male counterpart, the pollen, unless it can be isolated.

The significance of ES clearing and isolating of ESs is two fold:

The clearing technique could be useful in providing a clear picture of the ES elements before, at and after pollination, which enables the monitoring of ES elements during the fertilisation process. The technique also serves as an excellent tool for understanding of plant reproductive biology in general, understanding of the pattern of embryo development, and the manipulation of ovules, in particular. In the present study, the clearing technique was used in an attempt to trace fluorescent probes into the ES; however, the clearing process seems to have affected visibility of these probes. As stated by O'Brien and McCully (1981), the study of internal structure using cleared preparation is limited by light losses due to absorption by pigments and to scattering. They reported that the use of lactic acid, for instance,

destroys the cytoplasm, which must have an effect on the stability of the fluorescent probe loaded into the ES.

The enzymic maceration of ESs, on the other hand, is another essential tool of accessing female gametophytes, both toward embryo culture and the manipulation of individual protoplasts. However, special interest attaches to the role of the embryo culture method in overcoming the developmental block and breaking dormancy mechanisms which in most cases hinge on the inability of the enclosed embryo to grow (Raghavan, 1977). Several other important benefits can be distinguished. These include the application of more controlled conditions and new *in vitro* pollination procedures and an integrated procedure for culture conditions. The combination of *in vitro* culture methods in the new pollination methods to overcome pre- and post-fertilisation barriers should make it possible to obtain interspecific crosses more efficiently as showed in a study by Van Tuyl *et al.* (1991). However, difficulties remain; prolonged ES enzymic maceration, as carried out by Hu Shi-yi *et al.* (1985), led to partial digestion of the boundary wall of the ES and to the release of the protoplasts of the ES elements.



#### ***In vitro* ovule culture:**

Ovule culture has proved particularly rewarding in studying the behaviour of proembryos of certain plants where embryo excision and culture is not possible. The technique is considered effective in overcoming pre-fertilisation barriers (Rangaswamy and Shivanna, 1967). Having pollinated ovules from self-incompatible *Petunia axillaris*, the authors observed adult embryos and normal endosperm.

In the present study, it was observed that ovules cultured in MS10 and MS11 went brown and did not show any sign of regeneration, compared to MS12, in which no damage to the developing ovules could be seen. The browning of ovules cultured *in vitro* may be attributed to oxidation of polyphenols, which suggests that a physical and chemical deterioration of the tissues has occurred leading to necrosis.

In this study, it was clear that of the fifteen media tested, MS12 and Nitsch and Nitsch (1969) media supplemented with 50 g l<sup>-1</sup> sucrose and 9 g l<sup>-1</sup> agar were most appropriate for *N. tabacum* ovule culture. It can be concluded that a medium composed of MS (4.703g l<sup>-1</sup>) + agar (9 g l<sup>-1</sup>), sucrose (120 g l<sup>-1</sup>), NAA (0.1 mg l<sup>-1</sup>), 6-BAP (1.0 mg l<sup>-1</sup>), glutamine (400 mg l<sup>-1</sup>), Vit1 (0.1 mg l<sup>-1</sup>), Vit6 (0.1 mg l<sup>-1</sup>) is appropriate for *N. tabacum* ovule culture.

#### ⑨ ***In vitro* fertilisation:**

The technique of *in vitro* pollination and fertilisation has found application in overcoming sexual self-incompatibility, which was defined by Rangaswamy (1977) as 'a physiological barrier which prevents fusion of sexually different gametes, which are otherwise fertile, produced by the same individual of a heterosporous species'. The technique helps eliminate self-incompatibility zones by bringing together the pollen and the ovule under aseptic conditions on artificial nutrient media.

The aim of *in vitro* fertilisation could be fulfilled using various pollination techniques: stigmatal, intraovarian, and placental pollination, however; in this experiment, stigmatal pollination and

direct pollination of ovules cultured *in vitro* was used. The ultimate objective was to bring about gametic fusion and normal development of *Nicotiana tabacum* embryos *in vitro*.

Stigmatal pollination in *in vitro* cultured *Impatiens glandulifera* gynecia resulted in obtaining pollen tubes penetrating the ovule. However, no seeds developed. Further experimentation should be carried out over a range of chemical and physical culture conditions with the aim of producing seeds in *I. glandulifera*, as in *B. napus* pollen were easy to germinate and they have clearly shown the feasibility of loading of fluorescent probes as discussed earlier. The uptake of LY-CH by *B. napus* pollen tubes and its transport into the ovules is a clear indication of the feasibility of using plasmolysis/endocytosis as a path to load xenobiotics into pollen, and from there into zygotes.

The results shown by *N. tabacum* ovaries cultured and pollinated *in vitro*, as evident from the germination of embryos and emergence of seedlings, is an indication that the prospects for controlled fertilisation in this species are quite promising. The origin of these plants which are produced *in vitro* could be attributed to other sources than sexual seeds, i.e. somatic embryos formed from ovary wall, or parthenocarpic seeds, but the fact that no germination was obtained with the control treatment would justify the supposition that these plants were the product of sexual fusion.

Finally, since the degree of successful *in vitro* fertilisation in plants varies from one species to another, the following criteria need to be fulfilled for achievement of such a vital objective:



(1) The chemical, as well as, the physical conditions, for *in vitro* growth and development, of both the male and female gametophyte, should be simultaneously optimised.

(2) The requirements for growth of the zygote, and the resulting embryo, are quite complex. Therefore assessment of the nutritional requirements, however variable, is quite essential for post-fertilisation development and seed formation.

### ⑩ **Production of genetically uniform line:**

It has been reported that juvenile shoots grow much more quickly than adult shoots which need to be rejuvenated. Roest and Bokelman (1981) reported better shoot development with explants taken from the top than from the base of the shoots. The success rate of plants obtained from terminal buds was higher than from lateral buds (Hollings and Stone, 1968). Growth hormones, play an important role. The concentration of cytokinin and auxin, for instance, seems to be of paramount importance. It is desirable to use a low auxin concentration, together with a high cytokinin concentration, usually in the ratio 1:10 (Pierik, 1975). The rate of shoot proliferation is generally affected by numerous factors of which the chemical composition of the primary culture medium and the physiological state of the plant material are of major importance.

The results shown in Figures 30 & 31 described earlier reveal that the ability of plants to regenerate *in vitro* is determined by the genotype, nutrient media and the nature of plant material, i.e. the ability of plants to regenerate *in vitro* is species-specific as well as plant-organ-specific.

The Nitsch and Nitsch Basal Salt Mixture contains the concentrations of macro- and micronutrients as described by Nitsch and Nitsch (1969) and does not contain auxins, cytokinins or organics. This medium was originally developed for culturing haploid plants from pollen grains of *Nicotiana* and has also been adapted for the culture of many plants including *Asparagus* and *Chrysanthemum*. As with *B. napus*, whole pistils and pedicels are more capable of regenerating *in vitro* compared to culture of single nodes from the same plant. Therefore, the results described earlier reveal that the regeneration potential in plants varies according to the source of explant cultured. Similar research results support the finding expressed earlier, that plant micropropagation potential varies from one plant organ to another. For instance, adventitious buds could be formed from leaves in *Begonia* (Shigematsu and Matsubara, 1972), root sections of *Convolvulus* (Bonnet and Torrey, 1965), inflorescence sections of *Haworthia* (Majumdar, 1970) and cotyledon of *Biota* (Konar and Oberoi, 1965).

It was shown earlier that the subculturing of callus produced by *I. glandulifera* nodes, in solidified and liquid MS-based media was not effective with regards to further callus setting and/or subsequent regeneration. This could, in part, be attributed to the unsuitability of the physical and/or chemical environments, or in another part, to the effect of subculturing of the primary callus on reducing the competency for morphogenesis (Narayanaswamy, 1977). However, in exceptional instances, leaf tissue cultures such as that of tomato had undergone 30 passages in culture without losing organogenetic capacity for propagation on a massive scale (Padmanabhan et al., 1974).

The variation in response to micropropagating *I. glandulifera*, *B. napus* and *N. tabacum* described earlier and the difference in ability to regenerate between organs of the same plant (*B. napus*) could be explained by one or all of the following three hypothesis:

Firstly, the hormonal status of the explant which can affect both the direction and the rate of nutrient movement (Peel, 1974). Secondly the nutritional status, and thirdly, the association of specific growth substances with specific resource starvation (Trewavas, 1987). This author stated that 'auxin and gibberellin may be associated with carbohydrates and amino acid starvation in etiolated and green plants, cytokinin with nitrogen starvation, abscisic acid with water, and ethylene with ATP (O<sub>2</sub>) starvation. Therefore it can be concluded that both hormonal and nutritional status of a plant organ at any one time affect the plant organ's ability to regenerate.

#### **Recommendations for further work:**

- It was shown earlier that this study has revealed the feasibility of loading xenobiotics by plasmolysis/endocytosis (LY-CH, fluorescein), and by enzymic cleavage (FDA) via germinating pollen. However, the following criteria would describe the ideal experimental plant which satisfies most, if not all, aspects of the research objectives outlined before:
  - (1) A plant with pollen that are easily germinable *in vitro*,
  - (2) A plant of which viable embryo sacs could easily be isolated and/or cleared,
  - (3) A plant of which whole ovaries, ovules and/or embryo sacs could be cultured *in vitro*,

- (4) A plant which is capable of regenerating *in vitro* as a genetically uniform line is desirable, for elimination of any variability that might affect the consistency of results,
- (5) A plant in which the chemical and physical requirements for *in vitro* growth, for both the male and female gametophytes, are simultaneously satisfied.
- It can be predicted, from the feasibility of loading xenobiotics expressed earlier, that the ideal experimental plant can be tested for loading of biologically active material and foreign DNA/genes using the plasmolysis/endocytosis technique.
  - It was beyond the scope of this study to assess the enzymic status of pollen, however, it would be necessary to assess it, before a concrete conclusion is drawn on the significance of the pollen's physical and/or physiological dimensions with respect to their effect on viability.
  - It can be recommended that to assess plants' regeneration ability it would be most valuable to first assess the hormonal status of the plant material *in situ* (by extracting growth regulators and looking for their levels in the plant, particularly the level of free auxin), the nutrient status and the association of specific growth substances with specific resource starvation could be taken as the key criteria to reveal the potential of the particular plant organ to regenerate *in vitro*.

## CHAPTER FIVE

### SUMMARY AND CONCLUSIONS

The overall aim of this research project was to investigate the possibility of using pollen as a vector for transporting extracellular substances to the site of gamete fusion in the embryo sac. The following represents a summary of the experimental results from the individual steps in the protocol for this procedure:

#### ① Pollen culture *in vitro*

It can be concluded that a simple sucrose/boric acid solution provides the necessary nutritional requirements for *Impatiens glandulifera* and *Nicotiana tabacum* pollen germination and pollen tube growth *in vitro*. The addition of calcium, potassium and magnesium promoted *in vitro* pollen tube growth.

The slower pollen tube growth which resulted from the increase in the concentration of sucrose provides evidence of the importance of these compounds as osmotic regulators in addition to their nutritional role. Therefore, it is vital to develop a culture medium with approximate similarity between the osmotic concentration of the medium and the pollen grain in order to safeguard pollen grains from bursting and at the same time allow for uninterrupted pollen tube growth *in vitro*.

The response of pollen tube growth varied between liquid and solid culture media. Supplementing pollen culture medium with agar gave a slower growth rate compared to liquid culture media. Nevertheless, it facilitated further experimentation on germinated pollen including staining techniques for identification of callose plugs and monitoring of the behaviour of pollen nuclei *in vitro*. Solidified culture media is much more reliable in combating pollen bursting when compared to liquid media, possibly due to slower water influx into germinating pollen.

The use of the MicroScale Image Analyser as a tool for measuring pollen tube growth *in vitro* provided more precision and minimised the effort required to carry out the measurements of pollen tube growth *in vitro*.

**Effect of  
temperature on  
pollen growth *in  
vitro***

It can be concluded that optimum pollen tube growth for *I. glandulifera* can be achieved at temperatures in the range of 4-24 °C. Temperatures higher than room temperature resulted in a higher frequency of bursting of pollen and retarded the growth of pollen tubes. The effect of temperature on pollen tube growth may be attributed to its effect on hydration status, and subsequently, the osmotic concentration of the culture medium, and to its effect on the rigidity of the pollen tube wall and on metabolic processes.

**Effect of  
humidity on  
pollen growth *in  
vitro***

The stimulatory effects of increasing humidity in the atmosphere above the pollen germination medium on pollen tube length and growth rate are interesting.

The use of elevated humidities in the controlled hydration of pollen is known to be important in improving pollen germination and avoiding bursting due to excessive rates of water influx into hydrated pollen. The importance of atmospheric humidity in the regulation of pollen tube growth in an aqueous or agar-based medium *in vitro* is less clear. One possible explanation is that the humidity gradient between the liquid surface and the atmosphere above regulates evaporation or creates an osmotic gradient within the surface layers of the pollen germination medium, which then provides a more favourable environment for pollen tube elongation. If, as seems likely, the metabolic requirements of tube growth vary during growth, it may be that a gradient of changing concentrations established at the interface between the pollen germination medium and the atmosphere is required for optimum pollen tube growth *in vitro*.

A detailed study of the changing physical and nutritional requirements of pollen during growth is now required, to further explain the observations made in the present study.

## ② Pollen viability

The results obtained in this study reveal shortcomings on the most widely used method for assessing pollen viability, the fluorochromatic reaction test which demonstrates the activity of esterases and the state and permeability of the plasmalemma. This is evident from the lower fluorescence intensity obtained after incubation of pollen at low temperatures (4-8°C). However, as reported earlier, longer pollen tubes were obtained under these temperatures compared to higher temperatures, which indicated that the fluorescence intensity brought about by the FCR reaction showed a negative correlation on pollen growth in terms of tube length.

In addition to that, some densely FCR positive pollen failed to germinate in comparison to lightly FCR positive which did germinate. This agrees with some other researchers findings, in which pollen germination percentages lower than FCR values were reported. More support for this argument could be obtained from studies mentioned earlier in which FCR negative pollen grains including FCR positive sperm cells were noticed both for fresh and stored pollen.

The response of pollen to staining by CFW revealed a negative correlation between fluorescence intensity and incubation temperature, which seems more likely to be a reliable indication of pollen viability with the increase in incubation temperature. Therefore, the penetration of CFW and the subsequent fluoresce in pollen grains is due to a disrupted plasmalemma or cell membrane, a clear distinction between germinable and non-germinable pollen.

## ③ Pollen developmental state and monitoring of pollen nuclei

This study reveals the reliability of Feulgen staining technique, aniline blue and DNA specific probes (e.g. DAPI) as valuable tools for assessment of pollen developmental state, the understanding of which is undoubtedly essential for understanding the biology of pollen in general and the fertilisation process in particular.



These techniques were successful with pollen from *I. glandulifera* and could be applied to other plant species as well. The technique clearly shows cell division, microspore arrangement and the timing of division of pollen nucleus into vegetative and generative nuclei. Additional and valuable information on the position and functional importance of the vegetative nucleus could also be obtained using this technique.

④ **Identification of pollen tubes**

It can be concluded that the use of 1% w/v aniline blue solution dissolved in 1M tri-potassium phosphate is an effective method by which pollen tubes were successfully identified and callose plugs were made clearly visible using blue violet excitation.

It can also be observed that there are three different type of callose plugs in *I. glandulifera*, of which one is most common. The pattern of callose plug formation has also been observed as species-specific, where the distance between callose plugs in *N. tabacum*, for instance, is always the same, as compared to *I. glandulifera* where callose plugs are widely dispersed without a specific pattern.

The emergence of four pollen tubes at a time from *I. glandulifera* pollen is due to the fact that the four germination pores have been activated at the same time, which is not a normal feature of the species, and is mainly attributable to the effect of the culture medium.

⑤ **Loading of fluorochromes into pollen by Plasmolysis /endocytosis**

This study provided clear evidence of uptake of LY-CH by pollen after plasmolysis and deplasmolysis in the presence of the probe. LY-CH does not appear to diffuse across the plasmalemma; therefore the most likely mechanism by which this probe was taken in was through endocytosis

It can also be concluded that fluorescent probes, e.g. FDA, can be enzymically cleaved in pollen without affecting pollen ability to germinate and produce pollen tubes carrying the enzymically cleaved probe.

The significance of such findings opens the door for manipulation of pollen through endocytosis using exogenous material. This allows the use of pollen as a natural vector to deliver biologically active material to the ES without having to go through complex procedures of gamete isolation and/or microinjection techniques.

**⑥ Loading of fluorochromes into plant reproductive cells via the vascular system**

Fluorochromes were loaded into plant cell via the vascular system to avoid the potentially damaging plasmolysis/deplasmolysis procedure which is necessary for loading fluorochromes into cell by endocytosis. It can be concluded that fluorescent probes such as LY-CH, CFW and fluorescein can be loaded into plant ovules apoplastically and symplastically via the pedicels of *I. glandulifera*, *N. tabacum* and *B. napus*. Traces of LY-CH were seen in the style and stigma cells of *N. tabacum*.

It was observed that fluorescein loaded via the pedicel of *I. glandulifera* before anther opening resulted in uptake of the probe by pollen grains. When the pollen was germinated, the probe was also clearly visible in the pollen tubes. It can therefore be concluded that manipulation of pollen grains is also possible via the pedicel route, especially at earlier stages of growth before formation of the sporopollenin.

The use of fluorescent probes also reveals information on translocation routes which could help in understanding the pathway of the nutrients in the developing ovule, which is very limited at present.

**7 Manipulation of ovaries/ovules and clearance of ESs**

The embryo sac is much more difficult to manipulate than pollen due to its inaccessibility. Each of the three techniques used in clearing embryo sacs reveals some information, and the use of all the three techniques is important if a clear picture is to be drawn.

It can be concluded that the use of the fixing medium and clearing technique described in the methods section has proved satisfactory in clearing embryo sac in *I. glandulifera* and *N. tabacum*. However, not all elements could be seen simultaneously as DIC optics views one optical plane at a time. Furthermore, the fixing and clearing technique affected the fluorescent probes loaded, and must therefore be considered unsatisfactory methods for detecting these probes in the embryo sac.

On the other hand, the fixing, dehydrating and embedding of plant ovules in LR White can be considered a satisfactory technique in assessing uptake of fluorescent probes into embryo sacs. This is evident from the traces of LY-CH detected in *I. glandulifera* embryo sac after loading of the probe via the pedicel.

It can be concluded that the enzymic maceration technique was successful in isolating embryo sacs from *I. glandulifera* ovules. These ovules were FCR negative, and were therefore considered non-viable. The application of enzymes affected tracing of the fluorescent probes. The technique could be useful in embryo sac culture if the type and optimum concentration of enzymes, as well as the time of ovule maceration, are achieved.

**8 *In vitro* ovule culture**

It can be concluded that a medium composed of MS, sucrose, NAA, 6-BAP, glutamine, and Vit1 and Vit6, is appropriate for *N. tabacum* ovule culture. Other MS-based media did not seem to be as suitable.

The role played by growth hormones is essential in enhancing ovule development *in vitro* and minimising damage could be attributed to either or both of the following two reasons. Firstly, they promote growth, therefore, making plant tissue a sink that draws uptake of nutrients, and secondly by facilitating transport of nutrients.

⑨ ***In vitro*  
fertilisation**

It is evident from the results shown by *I. glandulifera* ovaries pollinated *in vitro* that this species did not show any ability to set seeds *in vitro* under the experimental conditions employed here.

However, it can be concluded from the results shown by *N. tabacum* ovaries cultured and pollinated *in vitro*, as evident from the germination of embryos and emergence of seedlings, that the prospects for controlled fertilisation in this species *in vitro* are good. The plants resulting from *in vitro* pollinating of cultured *N. tabacum* ovaries can be considered a product of sexual seeds as no such response was obtained from the unpollinated ovaries.

This project was designed as a feasibility study, to investigate the difficulties involved in performing the individual manipulations needed for inducing pollen to take up xenobiotics and transport them to the embryo sac. For reasons of convenience and logistics, three different plant species were used to test the steps in the protocol. The procedures must now be repeated in sequence, using a single plant species, to achieve the desired goal of using pollen as a vector for xenobiotics in the fertilisation process. The criteria for an ideal experimental plant for this procedure is outlined on section (4.6).

At the beginning of this study it was decided that genetically uniform material should be used wherever possible, in order to maximise the repeatability of the results. The following summarises the results obtained from attempts to micropropagate *Impatiens glandulifera*, *Brassica napus* and *Nicotiana tabacum*.

⑩ **Production of genetically uniform line**

Having used a variety of culture media, it can be concluded from the experiments aiming at producing a genetically uniform line that plant species vary in their capability to regenerate *in vitro*, as do different parts of the same plants. This is evident from the negative response of *I. glandulifera* as compared to *B. napus* and *N. tabacum*. However, when the single node culture and whole ovary culture were used with the latter species, successful rooting and shooting *in vitro* resulted from pedicels of *B. napus* and single nodes of *N. tabacum*.

It can be concluded that the ability of plants to regenerate *in vitro* is species-specific as well as plant-organ-specific. The hormonal status of the plant organ, the nutrient status and the association of specific growth substances with specific resource starvation could be taken as the key criteria to reveal the potential of the particular plant organ to regenerate *in vitro*. Therefore, the understanding of the plant's hormonal and nutritional status would reveal useful information about the potential of a plant organ to regenerate *in vitro*.

◆ **Concluding Remarks**

The various aspects of the present study have revealed that the plasmolysis/endocytosis of pollen would be a feasible route of loading biologically active or inactive material, as expressed by the application of fluorescent probes in this study. Such a significant finding would open the door for manipulation of pollen through plasmolysis/endocytosis using exogenous material; a step forward in the techniques employed in the manipulation of the male and female reproductive systems of flowering plants. It would be necessary, however, for research to be carried out to achieve this final goal.

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