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Techniques for genetically engineering lily pollen /

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TECHNIQUES FOR GENETICALLY ENGINEERING LILY POLLEN

A Thesis Presented

by

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ABSTRACT
TECHNIQUES FOR GENETICALLY ENGINEERING
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Genetic engineering of plants frequently involves the use of tissue culture. Tissue culturing of plants is time consuming, expensive, and in many cases increases the chances for mutations (somaclonal variation) to occur. Using pollen as the material to genetically engineer would surpass the need for these tissue culture stages. This present study explored techniques for pollen transformation. Electroporation of pollen, anther injection, and microinjection of pollen were analyzed using lucifer yellow, a fluorescent dye, and various plasmids expressing reporter and selectable marker genes. In the analysis of these techniques, problems were encountered at various stages in each of these methods. Optimal parameters for each of these techniques were establish employing Lucifer yellow. However, no expression of foreign DNA was observed with any of the techniques analyzed.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Transgenic plants via genetic engineering will be the practiced technology for plant breeding going into the 21st century. Some of the ground work for an economically feasible system for developing transgenic lilies and other monocots will be examined. Previous studies have been very encouraging for the creation of a methodology utilizing transgenic pollen.

The project objective was to establish a highly efficient, repeatable, and relatively economical methodology for introducing desirable genes into a wide variety of plant species. The emphasis is on horticulturally important plants, such as lilies. However, the system, once perfected, should be adaptable enough to accommodate a diversity of horticultural plants and agricultural crops.

The methods and parameters involved to transform lily pollen with exogenous DNA are studied in this thesis. The transformation of pollen has many advantages over traditional methods. Pollen transformation would not be limited by host range and would not require protoplast regeneration or tissue culture procedures. Tissue culturing is time consuming and expensive, and the culturing of many plant species is extremely difficult. Pollen collection, storage, and germination is relatively easy in comparison.

This present study explored techniques for pollen transformation. Electroporation of pollen, anther injection, and microinjection of pollen, along with screening techniques were analyzed. A Fluorescent dye was employed to determine the appropriate parameters for uptake of DNA. The main parameters analyzed for the electroporation technique were field strength of pulse, pulse duration, electroporation media, concentrations of pollen and DNA, lengths of pollen tubes and pollen viability. The anther injection parameters to be analyzed mainly

consisted of studying the correlation of bud length to pollen developmental stages. For pollen microinjection the parameters worked out consisted of the techniques involved with fixation and micromanipulation of the pollen.

Screening methods were examined to determine the best and most efficient system and detection for the exclusion of pollen that did not incorporate the target genes. This will be extremely beneficial in increasing the yield of the desired hybrid seedlings. Screening at the seed stage is more time consuming and costly.

The main goal of this work was to utilize the highly fluorescent dye, lucifer yellow, and to determine the parameters that were most efficient for incorporation of the dye into pollen, thereby establishing optimal conditions for creation of transgenic plants. Eventually other projects will attempt to add a beneficial foreign gene to this optimal transfer system, and test the progeny transgenic plants for expression and effectiveness of the useful gene product. After a model system with lilies is demonstrated, the research would be expanded to include other floriculture plant species and desired target genes such as disease resistance, insect repellency, environmental tolerance, increased growth and yield, desirable phenotypic traits, etc.

CHAPTER II

LITERATURE REVIEW

Improvement of plants through the use of genetic engineering techniques is necessary to keep pace with ever-increasing consumer demands. Classical plant disease and plant breeding research alone cannot provide applicable solutions in a desirable time-frame (Goodman et al., 1987). The most ecologically and probably most sound solution for providing plant disease resistance, high quality and aesthetic plants, and increase in yields, is to have these qualities be inherent in the plant itself. Plant molecular genetics research has led to the isolation and cloning of many genes of potential use. There have been some successes in applying these. A gene coding for the coat protein of tobacco mosaic virus (TMV) has been cloned into tobacco plants, and the resulting transgenic plants are resistant to TMV (Abel et al.,1986). The vector used for transfer of the TMV DNA into plants is one of the most successful vectors to date, the tumor inducing plasmid from the bacterium, *Agrobacterium_tumefaciens* (Benfey and Chua, 1989). However, there are many severe drawbacks to this delivery system for example, it is not applicable to very important monocotyledonous plants such as lilies, iris, amyaryllis, grasses, corn and cereal crops. Development of a DNA delivery system to include monocots as well as dicots is essential.

Advanced tissue culturing methods have been necessary for the successful uptake of DNA other than *A. tumefaciens*-related plasmids. Most of these studies utilized plasmids containing various enzymatic reporter or antibiotic marker genes controlled by the promoter from the cauliflower mosaic virus (CaMV). Transformation of plant cell protoplasts have been possible using enhancers such as polyethylene glycol (PEG) (Kruger-Lebus and Potrykus,1987; Negrutiu et al.,1987). Higher frequencies have been achieved utilizing electroporation of protoplasts

(Fromm et al., 1985). DNA has even been shot into suspension cultured cells by a microprojectile accelerator (Klein, et al., 1988) and foreign genes thus delivered were expressed. However, tissue culture methods are labor intensive and very expensive. Also, tissue regeneration into whole plants is still not possible with some species.

A natural DNA delivery system for most plants does exist. Pollination of plants results in seeds that contain genetic information from both male donor and female recipient. If beneficial foreign genes were introduced into pollen, the natural DNA delivery system could then be exploited. Pollen has shown itself to be an excellent system whereby plants may be transformed with agronomically useful genes (Lou and Wu, 1988; Hess and Dressler, 1984, 1990; Matthews et al., 1990). Not only is pollen apparently susceptible to transformation but unlike protoplasts, there is no need to induce regeneration since the resultant pollen can be used in normal pollination and fertilization. Transformation is frequently accomplished by microinjecting DNA into cells (Gasser and Fraley, 1989). However, mortality among injected cells is high and, since each recipient cell must be individually injected, only relatively small numbers of cells can be treated.

Limitations to microinjection may apparently be overcome by taking advantage of a unique feature of pollen mother cells. In the anthers of angiosperms, these cells, when about to undergo meiosis, are linked together by an extensive system of cytoplasmic bridges (Heslop-Harrison, 1966,1972). The function(s) of these cytoplasmic bridges is unknown, but possibly they serve to maintain synchrony between meiocytes (cells about to undergo meiosis). Whatever the actual functions, these bridges may be useful in improving the microinjection method of transformation. Injected into the pollen mother cells foreign DNA should be able to transfer through these cytoplasmic bridges, from one pollen mother cell to others. Thus, even if the pollen mother cell originally injected is damaged by the injection,

adjacent cells, still undamaged would be good candidates for the uptake of foreign DNA. The efficacy of this method has recently been demonstrated by de la Pena et al. (1987). They injected plasmid DNA into the developing floral tillers of the diploid rye cultivar JNK. They speculated that transformation might occur in either the male or the female gamete, with DNA moving through intercellular cytoplasmic connections. Transformation was indeed indicated, in one case, by 7 out of 3023 seedlings (from 98 injections) and, in another, by 3 out of 1000 seedlings being resistant to the antibiotic marker kanamycin. However, only 3 of the resistant seedlings (from 4023 total seedlings) showed activity of a second reporter enzyme that had also been present on the injected plasmid. The reason that only antibiotic resistance was detected and not the reporter enzyme may be due to rearrangement of the introduced genes, position affects in the host genome, and/or possible orientation problems. Many antibiotic resistance transposons used as selectable markers can be independent of orientation (Berg and Berg, 1981), but this is certainly not the case for all genes. Since there is a strong possibility that the target DNA may be incorporated, but not expressed in some of the resulting seedlings, it is essential to explore methods of increasing the efficiency of transformation.

The timing of the microinjection is crucial. Prior to meiosis, sporogenous cells are interconnected by normal plasmodesmata, each approximately 250 A in diameter, and, in totality, representing less than 1% of the contact area between cells (Ottaviano and Mulcahy, 1989). However, early in the meiotic prophase, other and larger channels, up to 2.5 um in diameter, develop between meiocytes. The diameters of these are sufficient to allow the movement of organelles between meiocytes, and in *Lilium henryi*, these channels occupy over 10% of the area between meiocytes, nearly 24% in *Dactylorchris fuchsii*. Thus interconnected, the meiocytes then share a common cytoplasmic pool. Tapetal cells also become linked together by unusually large channels but meiocytes and tapetum are not

interconnected. Channels between meiocytes persist, in *Lilium henryi*, until metaphase I, when callose deposits isolate meiocytes from each other.

For *Lilium longiforum*, the timing for optimum microinjection is crucial and can be fairly accurately estimated from bud length (Erickson, 1948; and my own research), and confirmed by injection of fluorescent tracers (Stewart, 1978).

Electroporation consists of high intensity electrical impulses that are known to reversibly permeate membranes of mammalian, plant and microbial cells. This offers a unique method for introducing macromolecules into the cells that are normally impermeable to the cell membrane. Electroporation mediated gene transfer has been shown in both monocot and dicot plant protoplasts (Fromme et al., 1985),and the technique has been optimized to achieve gene transfer efficiency of about 2% in tobacco protoplasts. Mishra et al., 1987 have shown that various dyes can be taken up by electroporation of germinating pollen grains and has suggested that the introduction of large molecular weight molecules into pollen is possible without significant decreases in pollen viability.

Abul-Baki and Saunders et al., have shown that electroporation of germinating pollen grains of *Nicotiana gossei* take up foreign DNA under a variety of conditions, without detrimental effects on the viability of the pollen. By optimizing both the field strength of the electroporation pulse and the DNA concentration in the electroporation medium, up to 6% of the donor DNA can be taken up by the germinating pollen while maintaining a pollen viability of 90%. Field strength as high as 9 kV/cm could be applied to germinating pollen grains without detrimental effects on viability.

Fluorescent probes, injected directly into the cytoplasm, are being used increasingly as a means of studying intercellular transport in plant tissues. One essential criterion for such studies is that the probes should be unable to cross the plasmalemma and remain confined to the compartment into which they were

injected. Lucifer Yellow is a highly fluorescent dye with this distinctive attribute. Also, this fluorescent dye has a number of additional properties than the more traditional fluorescent dyes that makes it particularly suitable for microinjection as well as electroporation studies (Wright and Oparka, 1989). (1) It is highly soluble in water. (2) Its quantum yield does not vary between pH 1-10. (3) It is lipid insoluble. And (4) it is highly dissociated at physiological pHs.

Proper selectable and reporter genes also play a crucial role in transgenic studies. These are genes which can be conveniently monitored to confirm that the DNA vector has been taken up and expressed in the plant cell or pollen. B-glucuronidase (GUS), a traceable enzyme not native to most plant systems, is one of the most widely used reporter gene systems (Jefferson, 1989). This enzyme can be coupled with a CaMV promoter for use in plants. Chloramphenicol acetyl transferase (CAT) fused to a CaMV promoter is another traceable reporter gene system (Klein et al., 1988; Fromm et al., 1985). Also the luciferase gene has been successfully incorporated into transgenic plants (Ow et al.,1986) and causes the plants to fluoresce. This gene could therefore have application as a genetic marker. Other promoters more specialized for expression in pollen have more recently been isolated. The Zm13 promoter that was isolated from corn is a pollen specific promoter (Mascarenhas et al., 1990). This Zm13 promoter, when fused to the reporter gene GUS, has been found to work best in pollen for expression of foreign genes.

Antibiotic resistance selectable marker genes such as kanamycin resistance from the Tn5 transposon have also been very useful in plant transformation studies (Negrutiu et al.1987; Kruger-Lebus and Protrykus, 1987). Antibiotic resistance is not only useful in confirming that genes have been taken up and expressed, but also may serve to help eliminate the cells, pollen, or seeds that did not receive the resistance genes. Kanamycin was found to severely affect pollen tube growth of

normal tomato pollen, while significantly longer tubes were produced by hybrid tomato pollen grains expressing kanamycin resistance genes (Bino et al., 1987). Seed germination can also be affected by kanamycin (de la Pena et al., 1987). Another antibiotic resistance gene that could potentially be of use is hygromycin resistance (van den Elsen et al., 1985). It is clear that elimination of non-transformed pollen, and subsequent elimination of seeds that do not express antibiotic resistance, would certainly increase the chances of selecting a final transgenic plant.

Once a reliable and consistent method for transfer of genetic information into plants is perfected, full attention can be turned to the beneficial genes that might be incorporated into transgenic plants. As has always been the goal with classical breeding, traits such as disease resistance, insect resistance, pesticide tolerance, hardiness, yield increase, and desirable physical traits (ie. color, size and number of flowers, fragrance, plant height, etc.) would certainly be considered. However, unlike classical breeding, the source of the new genetic information need not even come from a plant at all. For example, some species of the nonpathogenic bacterium *Pseudomonas putida* exhibit an antibiosis reaction to a wide variety of plant pathogenic bacteria and fungi (Colyer and Mount, 1984). The gene responsible for this antibiosis, for example, may be useful if produced in the plant itself. There are many other genes from many other sources (Goodman et al., 1987) that can be targeted for beneficial transfer to plant host species. The potential of genetic engineering of plants is enormous, and the technology will be universally desired.

CHAPTER III

MATERIALS AND METHODS

Pollen

Planting Conditions

Pollen was obtained from Easter Lilies (*Lilium longiflorum* Thunb., cv. Nellie White and *Lilium longiflorum* Thunb., cv. Ace) which were kindly donated by Gloeckner and the Easter Lily Foundation. Lily bulbs were planted at weekly intervals in 6" pots in agromix number 2 soil (Fafard medium de croissance, Nouveau Brunswick, Canada). Gro-Tone Bonemeal 0-12-0 (Vigro Ind. Fairview, IL.) was added on top of the bulbs as a natural fertilizer to enhance flowering. Ban Rot (Sierra Crop Protection Co.,Milapitas, CA.) was sometimes used the first year (fall 1989-spring 1990) to prevent fungal bulb rots. This was applied immediately after planting in suggested levels by the manufacturer. The plants were grown under greenhouse conditions to the flowering stage.

Pollen Collection Drying and Storage

The pollen was harvested daily usually in the morning as soon as the flowers opened and the anthers were at least partially dehisced. The anthers containing the pollen were placed in an open petri dish on a laboratory bench for drying at least 24 hours before use. Direct pollen germination without prior drying was poor. Therefore, the drying stage was essential for proper germination to occur. After the pollen drying period, the pollen was removed by agitation from the anthers, then rehydrated for at least two hours but extending up to eight hours in a

rehydration chamber. The rehydration chamber consisted mainly of a closed Tupperware container with wet paper towels on the bottom. Although the pollen was best when used fresh after the drying and rehydration stages, it could be preserved after drying at 0^{o}C or at -20^{o}C.

Pollen Germination

Germination rates of pollen were determined by counting at least 200 pollen grains and scoring each pollen tube, that was at least the same diameter of the grain, as a successful germination. The pollen was germinated in Brewbaker medium, which consisted of 0.3 M sucrose, 1.62 mM H_3BO_3 and 1.27 mM $Ca(NO_3)_2$. The pollen was suspended in this medium and placed on a rotary wheel at 1 rpm at room temperature for approximately 1.5 hours.

DNA

Four different plasmids were used in this study. Approximately 30 ug concentrations of a specific plasmid were used in each of the appropriate experiments.

Plasmid pBI221

Plasmid pBI221 (figure 1) was purchased from Clonetech Laboratory Inc. (Palo Alto, CA.). This 5.7 kb construct contains a 3 kb region consisting of the Uid A gene encoding beta-glucuronidase (derived from pRAJ260, Jefferson et. al.,1986) and a CaMV 35S promoter that regulates expression as described by the supplier. The 2.7 kb backbone is a HindIII-EcoR1 fragment of plasmid Puc19. Selection

criteria in *Escherichia coli* was based on the ability to grow on culture plates containing ampicillin 100 ug/ml (Sigma Chemical Co.,St. Louis, MO.)

Plasmid pBI121

Plasmid pBI121 (figure 2) was also purchased from Clonetech Laboratory Inc. This 13kb construct contains the beta-glucuronidase (GUS) cassette with the CaMV 35S promoter in the binary vector pBIN19. This vector also contains both the right and left border regions of the *Agrobacterium* Ti-plasmid. This gene construct expresses high levels of GUS activity upon Ti-mediated transformation of tobacco cells. Selection criteria in both plant and bacterial cells is based on ability to grow on culture plates containing 50 ug/ml of the antibiotic kanamycin (Sigma).

Plasmid ZMc13

Plasmid ZMc13 (figure 3) was a gift from the laboratory of Joseph P. Mascarenhas. This is an approximately 5.5 kb construct containing the beta-glucuronidase (GUS) cassette fused to a pollen specific promoter (ZMc13) with a 3.2 kb Stratagene backbone (Stratagene La Jolla, CA.). Selection criteria in bacterial cells is based on ampicillin resistance at 100 ug/ml in culture media.

Plasmid PH1S

Plasmid PH1S (figure 4) was a gift from the laboratory of O.C. Yoder. This is a 6.7 kb plasmid containing the hyg B gene which encodes hygromycin B phosphotransferase fused to a fungal promoter that was isolated from *Cochliobolus heterostrophus*. The selection criteria in plant cells is based on ability to survive on

culture plates containing 100 ug/ml of hygromycin B (Calbiochem-Behring. La Jolla, CA.). In bacterial cells selection is based on resistance to culture plates containing 100 ug/ml of ampicillin.

Plasmid Digestion and Precipitation

The plasmids were always linearized without exception for all experiments utilizing DNA and were checked for proper digestion on a 0.7% agarose gel (figure 5). The plasmids pBI221, pBI121, and ZMc13 were all digested with the enzyme EcoR1 (Bethesda Research laboratory Co., Gaithersbury, MD.). The plasmid pH1S was digested with the enzyme SalI. The digestion mixture for obtaining linearized DNA was as follows; 100 ul DNA (30 ug), 10 ul of the appropriate 10 X buffer system, and 5 ul of the appropriate enzyme. The digestion mixture was then placed in a dry block heater (Thermostat Model 5320 Hamburg, Germany) set at 37^{o}C for two hours.

The digestion enzymes were then removed by an ethanol precipitation. Two volumes of 95% ethanol were added to the digestion reaction and gently mixed. The mixture was then placed in a -70^{o}C ultrafreezer for 30 minutes for precipitation of the DNA. After removal from the ultrafreezer the reaction was centrifuged in a Brinkmann eppendorf microfuge model 5415, (Westbury, NY.) for 10 minutes at 12,000 xg, at 4^{o}C. The ethanol was then decanted from the DNA pellet and the pellet allowed to air dry. Then the pellet was resuspended in sterile 5mM Tris pH 8.0, .5mM EDTA (TE) or dH_2O. The linearized DNA was stored at 4^{o}C for the short term or at -20^{o}C for long term storage.

Plasmid DNA Isolation and Purification

BRL (Bethesda Research Laboratories Life Technology,Inc.) library efficiency HB101 Competent cells were used in generating colonies of *E. coli* containing the specific plasmids. These *E. coli* colonies were then used for large scale plasmid purification. The transformation procedure was accomplished according to BRL guidelines. The guidelines include the addition of 1-5 ul of DNA to the partially frozen HB101 competent cells which were incubated on ice for 30 minutes. Heat shocking the cells for 45 seconds at 42^{o}C, with an additional two minutes on ice followed. Cells were then grown up for one hour at 37^{o}C shaking at 225 rpm, and then diluted to a 1:10 solution. The dilution was then spread on liquid broth plates containing 100 ug ampicillin, and incubated overnight at 37^{o} C.

The large scale alkaline lysis plasmid miniprep procedure used is described by Birnboim and Doly (1979) and modified by Ish-Horowitz and Burke (1981). During this study changes were introduced in several steps. Briefly, cells were grown overnight in 250 mls liquid broth containing the respective antibiotic, at 37^{o}C at 250 rpm. Cells were spun down for ten seconds at 10 xg in a Sorvall/RC-5B refrigerated centrifuge (Dupont, Wilmington, DE.). Pelleted cells were resuspended in 16 ml glucose-tris-EDTA pH 8.0 by vortexing. Two volumes of cells were combined in one 250 ml centrifuge bottle. A volume of 64 mls consisting of fresh 0.2 M NaOH, 1% SDS were added and mixed well by swirling. A volume of 48 mls of ice-cold 5 M KOAc were added and mixed. The mixture, after 5 minutes on ice, was then centrifuged for 6 minutes at 10K xg. The supernatant was then poured into a fresh centrifuge bottle and 0.6 volumes of isopropanol was added and immediately centrifuged at 10K xg for 10 minutes. The isopropanol was then discarded and the pellet allowed to air dry for 10-20 minutes. The pellet was then resuspended in 9.5 ml TE and 10.47 g of CsCl. A volume of 0.48 mls of a 10 mg/ml Ethidium Bromide

(EtBr) stock was added to a 50 ml centrifuge tube and left on ice for 10 minutes. This mixture was then centrifuged for 10 minutes at 12K xg. The precipitated material was removed and the supernatant placed in polyallomer ultracentrifuge tubes (Beckman, Fullerton, CA.). The tubes were then sealed and placed in a Beckman Ultracentrifuge 75-TI angle rotor at 44K xg for approximately 40 hours. After the plasmid band was pulled from the ultracentrifuge tube, the EtBr was extracted by adding the same volume of isoamyl alcohol to the mixture separating into two phases. The plasmid DNA was then dialyzed in 2 liters of tris acetate buffer (pH 8.0), changing the buffer 4 times, approximately every two hours with at least one overnight dialysis.

Concentrations of the DNA were determined spectrophotometrically measuring the O.D. at 260 nm. Assuming 1 O.D. at 260 nm is approximately equal to 50 ug/ml. The concentration of DNA can be calculated by (O.D at 260 nm) X (the dilution factor) X (50 ug/ml/1 O.D 260 nm unit). To zero the spectrophotometer, 1 ml of the appropriate buffer was used as a blank.

GUS Assays

Three different assays were studied in determining the best method to detect GUS activity. These were the fluorogenic assay, the histochemical assay, and the spectrophotometric assay.

Fluorogenic Assay

The fluorogenic assay was carried out by first collecting the pollen by centrifugation at 100 xg for 1 minute. The supernatant was discarded and the pellet was resuspended in 700 ul of GUS extraction buffer (Jefferson, 1987) containing 50 mM $NaPO_4$, pH 7.0, 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% Sarkosyl, and 10

mM B-mercaptoethanol, thoroughly ground in a 1.5 ml tissue homogenizer and centrifuged at 2500 xg for 10 minutes. Both the supernatant fraction and the pellet fraction of the pollen extract were assayed for GUS activity in GUS extraction buffer at 37°C. The reaction mixture consisted of 500 ul 1 mM 4-methyl umbelliferyl B-D-glucuronide (MUG) (Sigma) in lysis buffer and 200 ul of the pollen extract. The reaction was stopped by the addition of 0.2 M Na_2CO_3 to bring the final volume to 2 ml. The activity was determined by using a long-wave UV hand held light box. A series of known standards of MU (4-methyl umbelliferyl) in 0.2 M Na_2CO_3 were used also as controls.

Histochemical Assay

The histochemical assay for GUS involves the substrate 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc), (Sigma). The substrate mixture consisted of 0.5 mM X-Gluc in phosphate buffer (50 mM $NaPO_4$, pH 7.0). The total pollen, plus pieces of anther tissue on each injected anther being studied, was assayed. This pollen and tissue was broken up (mashed) placed in an eppendorf tube and resuspended in 1 ml of Brewbaker medium. Then approximately 200 ul of the X-Gluc stock was added. The reaction mixture was then incubated overnight at 37°C. The activity was determined by the presence of an insoluble and highly colored indigo dye.

Spectrophotometric Assay

The spectrophotometric assay was carried out either by growing the pollen of previously injected anthers that were allowed to mature, for approximately 4 hours or allowing germination of the pollen that was previously electroporated, for at least 24 hours after the procedure. The pollen was collected in both cases by

centrifugation at 100 xg for one minute. The procedure for indirect antibody ELISA for Elisamate enzyme immunoassay in microwell plates was followed as instructed by Kirkegaard and Perry Laboratories, Inc. (Gaithersburg,MD.). Modifications to instructions were made as advised by KPL and suggested by GUS users manual. Briefly, after collection by centrifugation the pollen was ground in a chilled mortar and pestl containing 1 ml of phosphate buffered saline pH 7.2 and pelleted again by centrifugation for 7 minutes at 14,000 xg. The supernatant was removed and specific dilutions were made at 1:10, 1:50, and 1:100 using the phosphate buffered saline. Two hundred microliters of these dilutions, including undiluted samples, were placed into ELISA plate microwells in triplicate and incubated overnight at 4^{o}C. After removal of the sample, the extract was blocked with bovine serum albumin (BSA) and then treated with the primary antibody solution, rabbit anti-GUS (Clonetech Laboratories, Inc.) in a 1:500 dilution and incubated for one hour at room temperature. The wash procedure was then carried out 3 to 5 times, which included filling of each of the wells with 500 ul of 1X wash solution and rapidly inverting the plate to empty. The secondary antibody was then added to the wells followed by another wash procedure. The substrate, at the recommended concentrations of 1.0 ug/ml, was then added to the wells. After 10 minutes of incubation at room temperature the peroxidase stop solution was added. The plates were then read by an ELISA plate reader (Dynatech MR600 Microplate reader, Torrance, CA.) at 405 nm. The printouts from the automated plate reader were then analyzed to determine activity.

Electroporation

The general electroporation procedure was applied to most experiments involving electroporation except if noted. The pollen was weighed and placed in 1.5 ml eppendorf tubes. A volume of 280 uls of 10% Brewbaker media was then added along with 20 ul of salmon sperm DNA at a concentration of 1 ug/20 ul (Sigma

Chemical Co.). The sides of the eppendorf tubes were then tapped so that the pollen was in the suspension. The pollen was usually germinated for 1.5 hours in the eppendorf tubes on a rotary wheel (New Brunswick Scientific Co.model TC-8, Edison,NJ.) at the speed of one rotation per minute. The pollen was considered germinated when the length of the pollen tube was at least the diameter of the pollen grain. The germinated pollen samples were then placed in cuvettes (VWR Scientific, Boston, MA.). Then at least 30 ug of DNA or 1 ug of Lucifer yellow (Sigma) suspended in water or TE was added to the pollen suspension. The cuvettes were then briefly and gently tapped before insertion of the 0.8 mm electrode gap into the electroporation chamber. A direct current (DC) pulse supplied by a square wave pulse generator (BTX, Inc. model 200 Electro Cell Manipulator, San Diego, CA.) was then delivered across the 0.8 mm electrode gap. The pollen suspension was not disturbed for times ranging from 15 minutes to one hour at room temperature after electroporation to allow resealing of membrane pores. After the appropriate resting period the pollen suspension was placed back into sterile eppendorf tubes. The pollen was washed twice in germination medium and collected by centrifugation at 100 xg for one minute. Samples of 20 ul were then looked at under the microscope. In experiments using fluorescent dyes the pollen grains were then scored at this point using a Nikon Diaphot Microscope (Garden City, NY.). The pollen grains were scored as positive with uptake of the fluorescent dye while maintaining a normal pollen physiology as in the control pollen grains. The remaining pollen suspension was then placed back on the rotary wheel for continued germination. After 10-24 hours the grains were scored for viability. Pollen grains considered to have survived the treatment had increased tube length along with the normal pollen morphology.

Injections of Anthers

To determine the appropriate bud length corresponding to the first meiotic metaphase, buds at various lengths were examined. The buds were broken apart with dissecting needles and stained with acridine orange (Sigma) on microscope slides under the dissecting microscope. The developmental stage was then determined under a high intensity inverted microscope. A 1 ml Tuberculin syringe with a 26 G1/2 Precision-glide needle (Becton Dickinson & Co.,Rutherford, N.J.) was used for the injection of the anthers. Approximately 100-200 ul of fluid was injected into each anther until drops on the opposite side of the anther were formed. The anthers were allowed to mature before analysis.

Microinjection of pollen

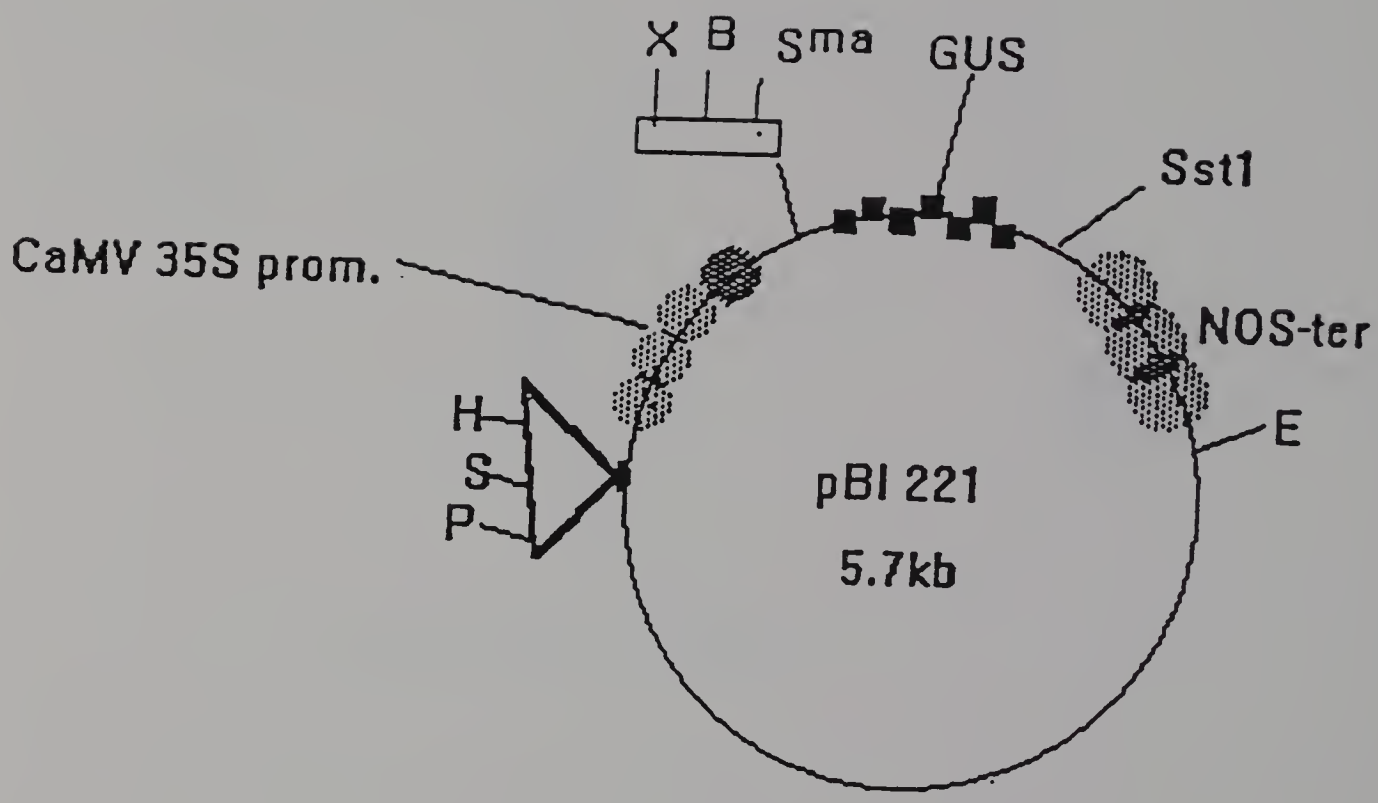
Microinjection of the pollen grains were carried out by again allowing the pollen to germinate approximately 1.5 hours in Brewbaker medium or until most of the pollen grains in the population had tube lengths as long as the diameter as the grain. A 1% agarose solution in Brewbaker medium was prepared and melted in a microwave, then placed in an 37 C incubator to maintain in liquid form. A 500 ul smear of this agarose solution was then made on a microscope slide using another microscope slide. Immediately following the agarose smear, a 20 ul drop of the pollen suspension was placed on top and spread out so that the pollen was partially embedded in the agarose solution before hardening. After the slide and pollen fixation were accomplished this preparation was set up under the micromanipulation scope. Micromanipulation of the pollen using pressure injection to inject the lucifer yellow/DNA was done using a VWR micro-pipette that was pulled by a Kopf Vertical pipette Puller (David Kopf Instruments, Tujunga, Ca.) and bevelled to the appropriate size opening by a Narishige Scientific Instruments Laboratories grinder (Setagava-KU Tokyo Japan). Injection of the pollen was located at the beginning of the pollen tube. The needle tip was pressed against the

pollen tube so that an indentation was obvious. Then the edge of the microscope was tapped so that the needle would then penetrate the tube. Careful injections were necessary so as not to permanently damage the pollen grain being injected. Five minutes after injection the needle was removed,being pulled out slowly so that the membrane was allowed to reseal correctly. The injected pollen tubes were then allowed to continue growth on the semi-solid media.

Pollen Screening

Screening pollen was carried out by using the antibiotics hygromycin B and kanamycin. The different concentrations of antibiotics were made up in Brewbaker medium. Pollen was germinated from the beginning in media with the differing concentrations of antibiotics. The experiment consisted of 4 mg of pollen in 1 ml of the appropriate media/antibiotic solution in an eppendorf tube. The pollen was then allowed to germinate for 5-6 hours. The pollen grains were then scored for ability to germinate in the differing concentrations of antibiotics.

Figure 1. Plasmid pBI221


X B Sma
GUS
Sst1
CaMV 35S prom.
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H
S
P
E
pBI 221
5.7kb

Figure 2. Plasmid pBI121

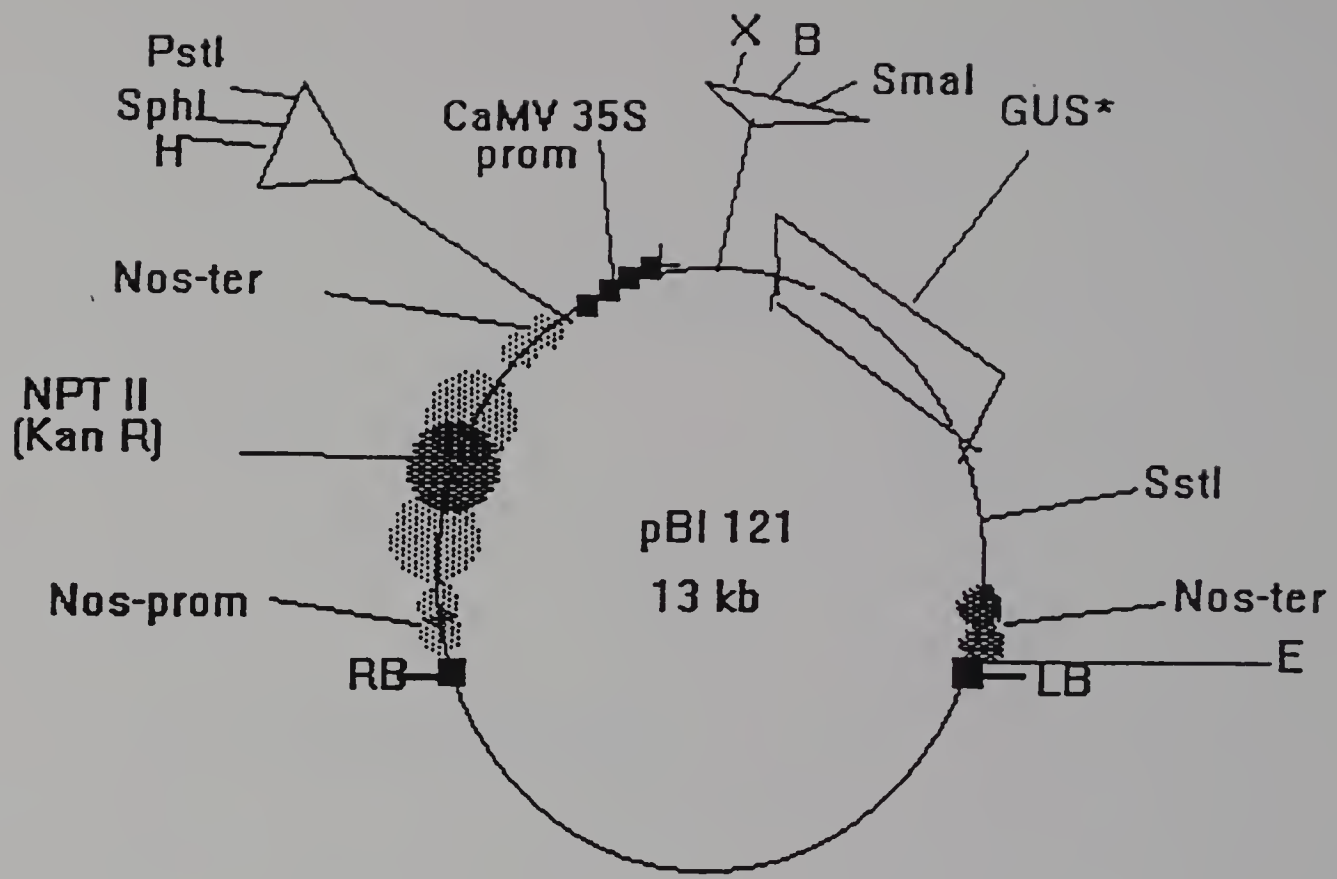
PstI
SphI
H
X
B
SmaI
CaMV 35S
prom
GUS*
Nos-ter
NPT II
(Kan R)
SstI
pBI 121
13 kb
Nos-prom
Nos-ter
RB
LB
E

Figure 3. Plasmid ZMc13

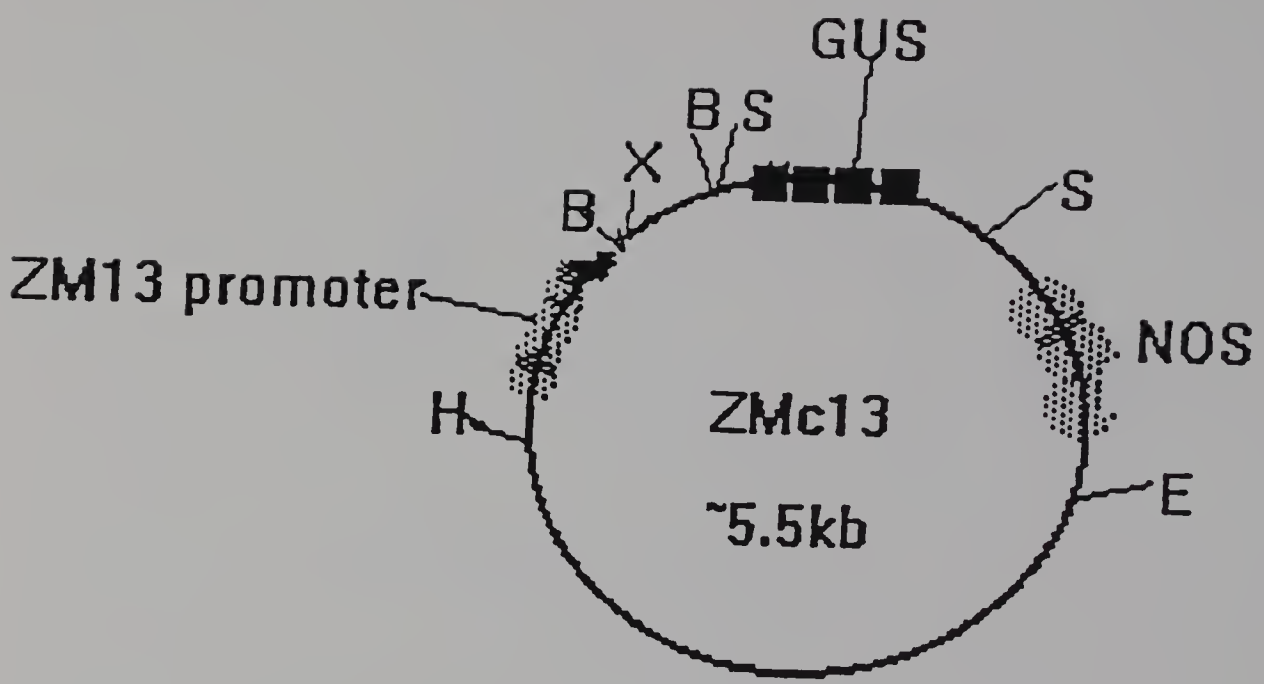
GUS
B
S
X
B
S
ZM13 promoter
NOS
H
ZMc13
~5.5kb
E

Figure 4. Plasmid PH1S

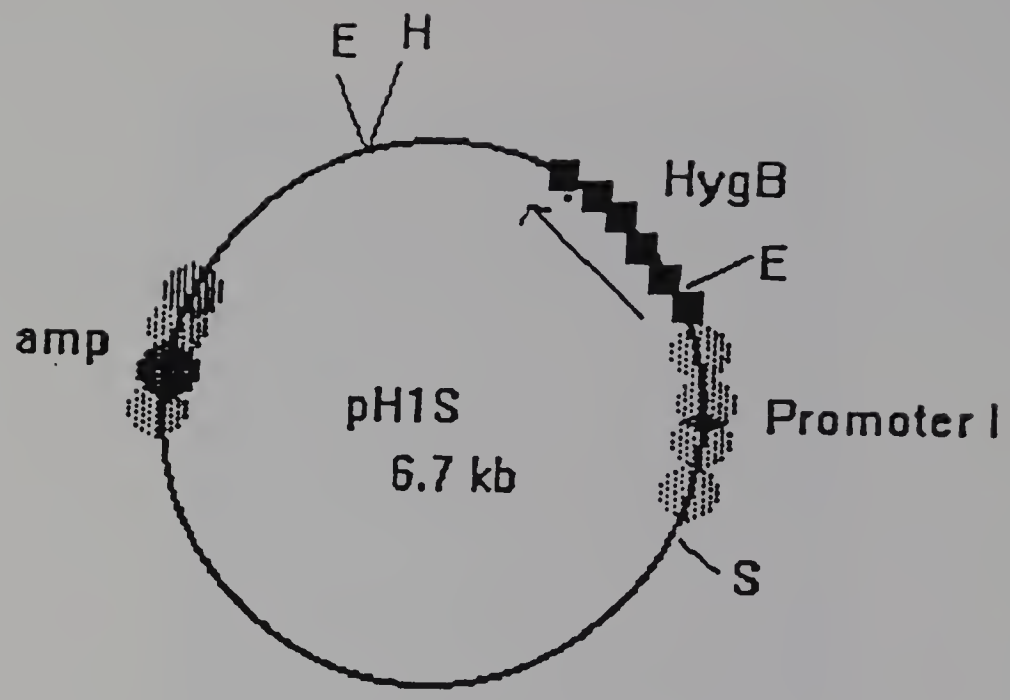
E
H
HygB
E
amp
pH1S
6.7 kb
Promoter I
S

Figure 5. A 0.7% agarose gel depicting plasmid pBI221 nonlinear (lane 4) linear (lane 5), and kb ladder standard (lane 6).

CHAPTER IV

RESULTS

Germination of Lily Pollen

At the start of this project, the percent germination of lily pollen was extremely variable from day to day. There are many different factors that can affect germination of pollen. Thus, it was very important to learn the conditions that would optimize the germination of lily pollen. Experiments were conducted to evaluate the best and most efficient method for pollen collection, storage, and germination. The results of many experiments indicated that the collection of the pollen was best during the morning or early afternoon immediately after the flowers opened and the anthers had unfolded and begun to dry (figure 6). There was a difference in the percent germination of pollen that had been collected a day or two later after anthesis occurred. The best method for drying and storage were also examined. The method that was found best for drying was determined to be at least 24 hours on a laboratory bench, however, longer than 72 hours of drying generally caused a reduction in the percent germination. Any type of storage, whether it be at room temperature, at 4 C, or at -20 C, for as little as one week reduced the percent germination. The most optimal situation for germination was also achieved by allowing a rehydration period of at least 4 hours prior to the addition of the germination media. Different percentages of sucrose were tried in the traditional Brewbaker's medium, but 10% sucrose always gave the best results. The results concerning the germination experiments of lily pollen indicated that timing of the collection, timing of the drying, and timing of the rehydration prior to the addition of the germination media were all crucial events in achieving an acceptable level of

germination for the experiments being conducted. Figure 7 depicts germinating lily pollen.

Screening Lily Pollen

Lily pollen was germinated in Brewbaker's medium along with different concentrations of the antibiotics hygromycin and kanamycin. This was the screening technique to be used to select for pollen that had incorporated and expressed foreign DNA. This selection technique would allow the transformed pollen to survive while killing the pollen not expressing the foreign DNA with the appropriate antibiotic resistance gene. Unfortunately this technique was only very useful in experiments using microinjection of anther tissue, where the pollen, if transformed, would express the resistance from the start of pollen germination. In the experiments that used electroporation and microinjection of pollen grains, the pollen was pregerminated prior to the transformation technique applied. Therefore, the screening technique using the antibiotics offered many problems to these systems. The first problem was, that time is needed for the foreign DNA to be expressed before adding the antibiotics. In both eukaryotics and prokaryotics, cells are usually given at least 24 hours and 8 hours respectively before expression is tested. Secondly, if the pollen was allowed expression time, of at least 24 hours after the appropriate transformation technique, the pollen tubes would be extremely long and fragile and the subsequent pollination would be extremely difficult.

There were differences in the amount of hygromycin and kanamycin needed to inhibit germination of nontransformed pollen grains. The antibiotics have different modes of action. Hygromycin is an aminocyclitol antibiotic produced by *Streptomyces hygrooscopus*, and inhibits protein synthesis by "interfering with translocation, causing mistranslation in both prokaryotes and eukaryotes" (Turgeon,

1987). Kanamycin interferes with the translation system of the 70s ribosomes as present in the mitochondria and the chloroplasts of eukaryotic cells (Bino, 1987).

The results of screening lily pollen with hygromycin (figure 8) suggest that at 20 ug/ml concentrations, 95% of the pollen was unable to germinate. Whereas at concentrations above this no germination occurred in all cases.

The results of screening lily pollen with kanamycin (figure 9) suggest that the pollen has a higher tolerance, at least initially, than tolerated with hygromycin. A concentration of 125 ug/ml prevents 98% of the pollen from germination, with concentrations over 125 ug/ml, no germination occurred.

Electroporation of Lily Pollen

Electroporation Parameters

The electroporation results with lily pollen was found to be in many cases extremely variable and, in some cases, difficult to interpret. Learning to score pollen for these electroporation experiments, and to be consistent throughout, was one of the first obstacles to overcome. Pollen grains considered to be positive, that is, having taken up the dye (lucifer yellow) and thus are consequently fluorescent are shown in comparison figures 10 and 11. However, one of the properties of lucifer yellow is that in dead or damaged pollen grains this dye accumulates and intensely fluoresces. Figure 12 depicts a dead pollen grain fluorescing, that was termed a "fried egg". The dead pollen grain is obviously abnormal with its cytoplasm pushed together making it relatively easy to recognize. Unfortunately, in some instances it was not as simple as this and a much closer examination was required to conclude if a grain was considered to be a positive or a negative.

Pollen germination was sometimes difficult to predict, and depended as mentioned earlier, on many parameters. Successful electroporation of pollen not

only depended on these parameters contributing to pollen germination but also on the electroporation parameters themselves, making this at times a somewhat difficult and variable system with which to work. For these reasons most of the time, at least three replicates were used in each experiment for each parameter being examined, and at least twice, the entire experiment was repeated with an average of three separate experiments.

In order to find the optimal conditions for uptake of high molecular weight material whether it be DNA or fluorescent dyes, many parameters were worked out. These parameters were determined by using lucifer yellow, a highly fluorescent dye.

Field strength was expressed as kV/cm and was influenced by the gap between the two electrodes of the electroporation chamber, amplitude of the applied pulse, and the resistance of the electroporation chamber. Figure 13 illustrates the electroporation setup. The electrode gap used in all experiments was 0.8 mm (figure 14). The formula used to calculate voltages into kV/cm = 800v/0.8mm = 10 kV.

The uptake of Lucifer Yellow by lily pollen grains that were previously germinated for one hour was influenced strongly by the field strength of the electroporation pulse. Figure 15 shows that the optimum field strength is 8.5 kVolts /cm. This is an average taken from three separate experiments. Although 8.5 kVolts was most consistently found to be the best field strength for dye uptake, in many experiments the optimum varied between 7.0 and 9.0 kvolts/cm. The pollen viability was also very much influenced by the field strength of the electroporation pulse. Figure 16 indicates that at the optimum field strength of 8.5 kvolts/cm for dye incorporation, the survival rate is approximately 60-65 %. The difference in survival percentages also remained the same regardless of which media was used for the electroporation procedure. Pollen was germinated in 10% Brewbaker medium in both cases then gently centrifuged and decanted; then 0.3M mannitol was added

to half of the samples and Brewbaker to the other half for the electroporation procedure. There were two reasons for using 0.3 M mannitol as an electroporation media. The first reason is that mannitol is a nonconductive media whereas Brewbaker is a conductive media. The nonconductive media in theory has less resistance, therefore the effectivity of the pulse is less impaired than with a conductive media. The second theory is that pulsing with Brewbaker as the electroporation media may affect the efficiency of uptake or increase cell death by allowing the materials in the external media to enter the grains. An over abundance of materials such as calcium, boric acid, and sucrose allowed into the pollen grains in high concentrations by the pores caused by electroporation, may lead to the death of the pollen grain. Although these theories are valid, the results in comparing both electroporation media basically show no difference in survival (figure 17). In comparisons made with dye uptake using both electroporation media no difference was found.

Pollen concentration was an important factor when considering resistance in the electroporation chamber. The results in figure 18, depict percent dye uptake with differing pollen concentrations. These pollen concentration experiments were conducted using a constant volume of media (300 ul), and field strength (8.5 kV/cm). The figure shows that at pollen concentrations of 4 mg/300 ul and 5 mg/300 ul the best percentage of dye uptake was achieved. However, when considering percent germination at these different concentrations, 4 mg/300 ul had the best germination and the tubes look more normal when compared with concentrations above 4 mg. In experiments examining both differing concentrations of pollen and field strengths this again showed that the 4 mg concentration with a field strength of 8 kV/cm was the most optimal condition for uptake of dye. The difference between 2 mg and 4 mg at the differing field strengths was sometimes only slight. At 8 kV/cm the percent dye uptake is 22 % for 4 mg of pollen compared

to 17 % for the 2 mg concentration of pollen (figure 19). Although this difference in percent dye uptake is 5 %, this was a consistent trend in most of the pollen concentration experiments.

Pulse width is the length of time a given field strength is applied to the sample which is being electroporated. Experiments were conducted to correlate the best pulse width with a given pulse to optimize dye uptake. Three different pulse widths were used; 60 usec.,80 usec., and 99 usec. with three different field strengths; 7 kV/cm, 8 kV/cm, and 9 kV/cm. The results shown in figure 20, confirmed that 8 kV/cm with a 80 usec pulse width was the optimal condition for dye uptake. If a 9 kV/cm field strength and a 99 usec. pulse width was used, there was only a 6 % dye uptake in the germinating pollen. This result was in part due to a large number of deaths caused from the electroporation procedure.

In order to know how much time was necessary to wait after the electroporation procedure before evaluation of the pollen, a resting period experiment was performed (figure 21). The theory of the resting period after electroporation is to allow the induced pores in the pollen grain time to reseal. This resealing time gives the pollen grain a chance to mend the damage caused by electroporation before any other manipulation is done, that may further damage the already fragile grains. The evaluation of the pollen grains only begins after the resting period is completed. The results in these experiments show that they were very slight differences in percent dye uptake corresponding to the differing resting periods. The trends in most of the experiments carried out show a very small but consistent increase in dye uptake by allowing a 60 minute time period of undisturbed rest after electroporation. This trend was also shown in many of the electroporation work previously published (Saunders, 1989).

The effect of temperature on dye uptake in pollen was examined. In several of the published reports temperature was said to enhances uptake of DNA in the

electroporation of plant protoplasts (Riggs, 1989 and Saunders, 1989). An experiment was conducted examining the percent dye uptake at differing temperatures at a constant field strength of 8.5 kV/cm (figure 22). The results of these experiments concerning the differences in temperature, showed it had little affect on percent dye uptake, but the general trend was that at 4°C the uptake was greater than at 24°C and 37°C.

Experiments were conducted to test the effect of multiple pulses at various field strengths and pulse widths. The results of these experiments confirmed other reports that large amounts of cell death occurs when the pulse is doubled. This death occurs even if very small field strengths are applied with corresponding short pulse widths.

Electroporation with DNA

Experiments were conducted using the optimal parameters interpreted from the fluorescent dye studies to now try to incorporate selected linearized DNA plasmids. The optimal parameters found were as follows; field strength of 8.5 kVolts/cm, a pulse width of 80 usec., a 60 minute resting period after electroporation, and a 4 mg pollen concentration. These were only some of the optimal parameters used in the DNA incorporation studies. Many other parameters were used to account for some of the variability, found in these studies. Some of the other parameters used involved higher and lower field strengths along with varying pulse widths. Enhancement techniques used in other studies were also pursued. Among these were, lower temperatures which are said to keep the induced pores open for longer periods so that high molecular weight materials such as DNA may enter more readily. The use of different media for both the pollen and the DNA were tried. The DNA was sometimes resuspended in Tris-EDTA (TE) which could end up clogging the induced pores or binding essential ions such as calcium that would inherently affect uptake and incorporation. Because of the possible affect of

TE, sterile distilled water was sometimes used for resuspension of the DNA. Also different preincubation time periods of the DNA with the pollen were tried from adding the DNA immediately before pulsing to adding it approximately two hours prior to the electroporation procedure. The addition of salmon sperm DNA which is suspected to act as a carrier DNA and may also have some protective affects preventing the cleavage of the foreign plasmid DNA was also tried. Approximately twenty-five different experiments were conducted using electroporation to incorporate the various plasmids available, but none of these experiments indicated any expression of this foreign DNA.

GUS Assays

Three different types of GUS assays were performed. The spectrophotometric assay, the histochemical assay and the fluorogenic assay were used to detect expression of the beta-glucuronidase gene that was present on three of the four plasmids used in these studies. The appropriate controls for each of the different assays were always used to determine if the assay was completed correctly.

In determining the best type of GUS assays to use much consideration was given to two factors. First the sensitivity of the assay, taking into consideration that size of the pollen sample was so small and the percent uptake was also relatively small in comparison to the more traditional sizes of the samples generally used in GUS assays for plant material. Secondly, the simplicity and speed was very important since so many samples were assayed.

The histochemical assay for GUS was generally the simplest to perform and the small size of the sample did not pose any problems. However, there was one problem that was discovered, that caused a number of false positives. In figure 23,

one can see the presence of the characteristic blue color indicating a positive in a sample of control pollen. This was later found to be due to the presence of GUS-like expression seen in many species of mature binucleated pollen (Plegt,1989 and Hu, 1990). Basically this assay was not used again in any of the other experiments.

The spectrophotometric assay for GUS expression was then explored. This assay turned out to be very time consuming taking at least three consecutive days to complete, and in addition was, very costly for materials. Furthermore with personal communications with James Saunders, it was decided that the assay was most likely not sensitive enough for this system.

The fluorogenic assay seemed to be the best candidate when enormously scaled down for the sample size and, was considered to be the most sensitive assay. The pollen samples was considered positive if the amount of fluorescence was over the basal expression that may have been picked up from the GUS-like activity in the control pollen.

The fluorogenic assay was conducted many times in search for the expression of the foreign DNA. Many different approaches were taken to optimize the assay. Longer incubation times were conducted with the substrate. The MUG stock was always made fresh, although it was not deemed necessary in the instructions. The samples were also assayed for expression at different time points ranging from 8-50 hours to see if any distinction in expression could be made. The results of this GUS assay along with the other assays tested, detected no expression of the foreign DNA.

Microinjection of Anther Tissue

The injection of lily anthers with both DNA and fluorescent dyes gave negative results . At the bud length of 10-14 mm lily anthers were injected. Figure 24 shows the stage of a lily plant when the leaves begin to fold back and expose the

buds at the proper injection stage. This bud length corresponded to 14 days before the first meiotic metaphase when the cytoplasmic channels are first formed between the meiocytes. The interpretation of some of the experiments where the fluorescent dye was injected into the anther tissue seemed to be positive at first , but upon closer evaluation the grains were dead. The results concerning this injection technique with both the plasmids pH1S and pBI221 did not show any positive results from the screenings done with hygromycin and kanamycin, and also the GUS assay performed.

Microinjection of Pollen

Injection of both the dye and DNA by pressure injection using a micromanipulator (figure 25) was very difficult and time consuming. The results of most of the grains injected resulted generally in bursting of the pollen grain or eventual death. The grains that were successfully injected had indicated no beta-glucuronidase expression when assayed.

Figure 6. Lily flowers (arrows) that have just opened. These flowers are at the optimum stage for pollen collection.

Figure 7. Germinating lily pollen in Brubakers medium.

Figure 8. The effect of various hygromycin concentrations on the percentage of germination of lily pollen.

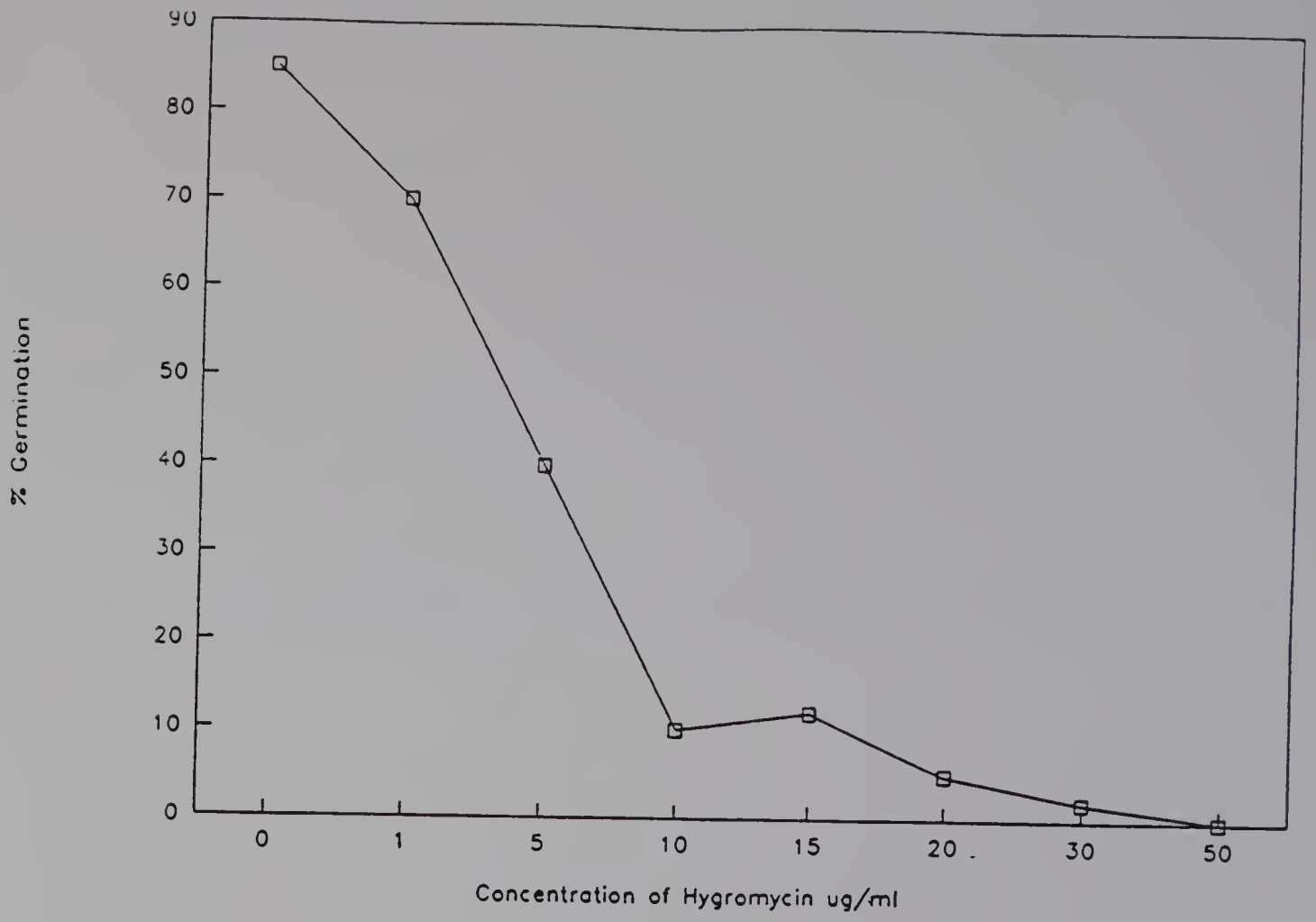
% Germination
90
80
70
60
50
40
30
20
10
0
0
1
5
10
15
20
30
50
Concentration of Hygromycin ug/ml

Figure 9. The effect of various kanamycin concentrations on the percentage of germination of lily pollen.

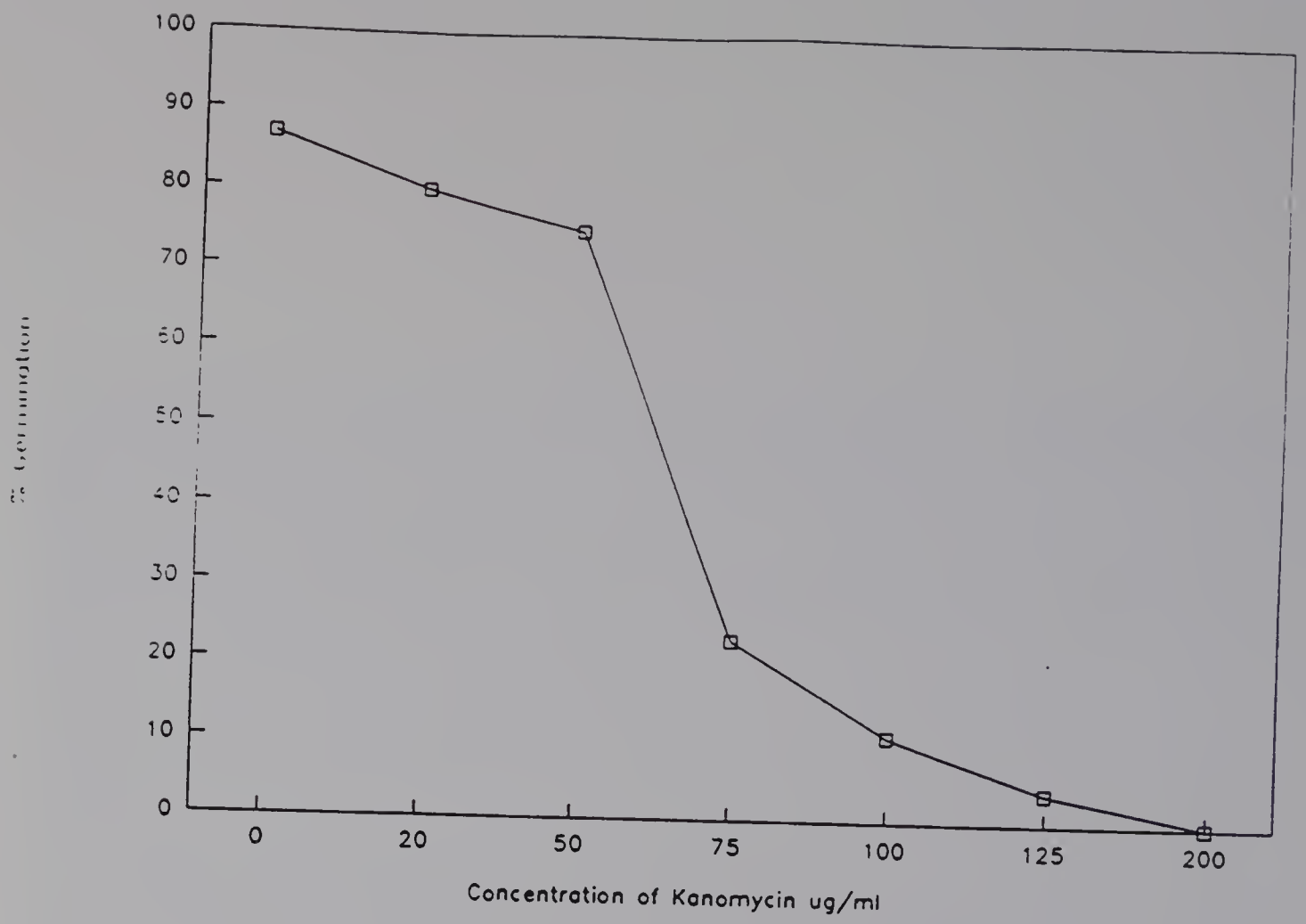
% Germination
100
90
80
70
60
50
40
30
20
10
0
0
20
50
75
100
125
200
Concentration of Kanomycin ug/ml

Figure 10. A fluorescent (lucifer yellow) and a nonfluorescent lily pollen grain.

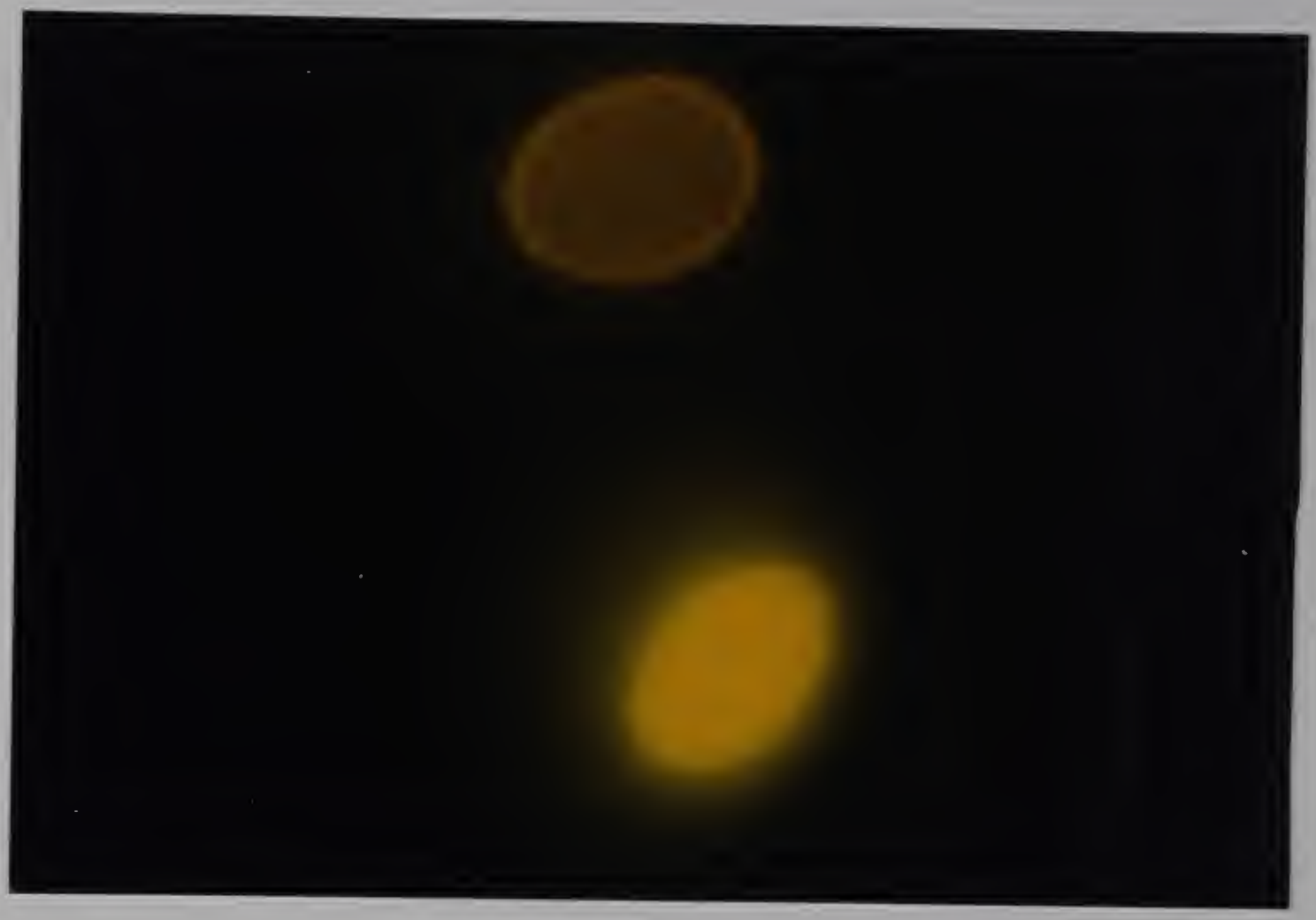

Figure 11. A fluorescent germinating lily pollen grain containing lucifer yellow and a nonfluorescent grain.

Figure 12. A florescent, dead lily pollen grain.

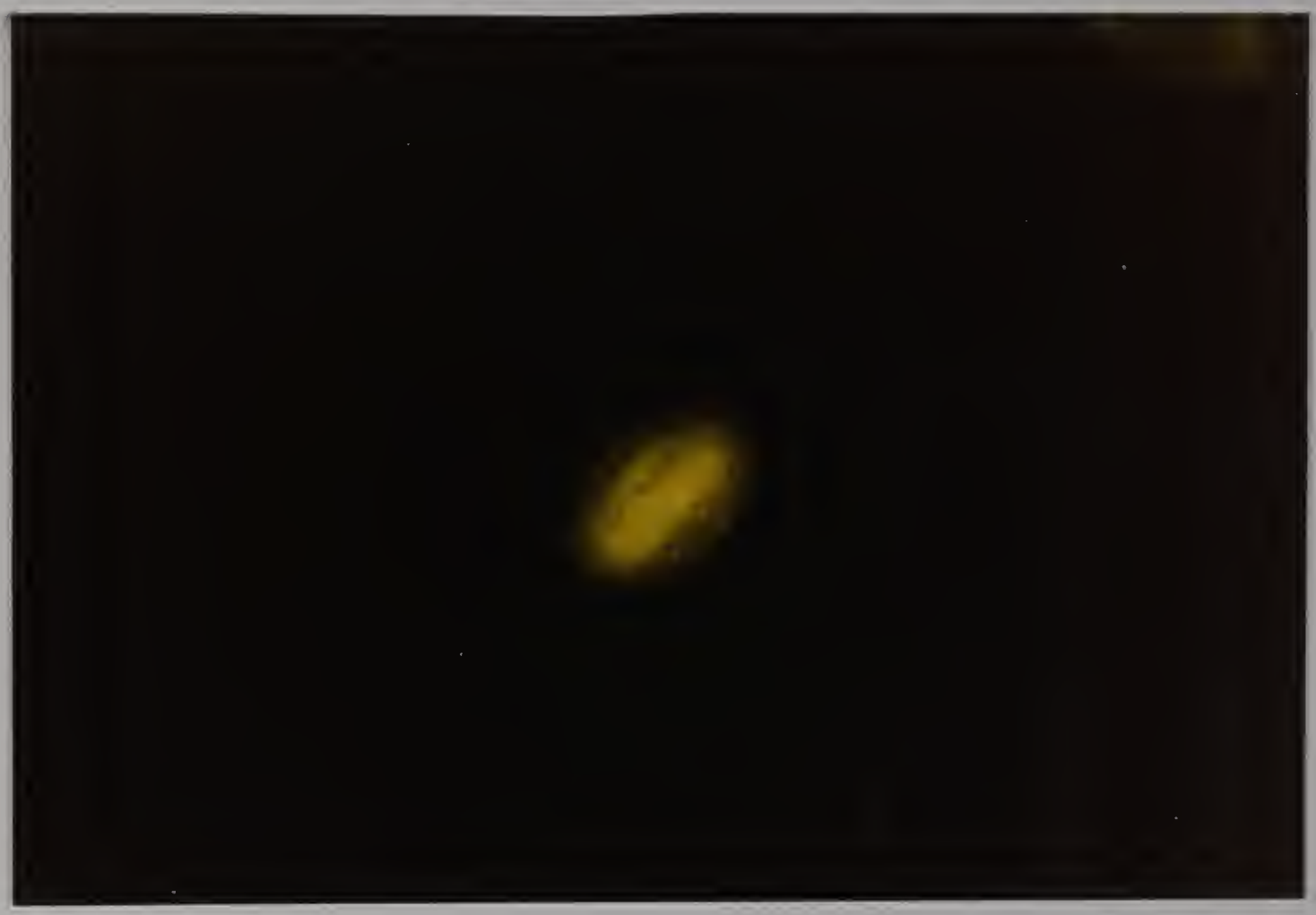

Figure 13. The BTX Electro Cell Manipulator used in the electroporation of pollen.


BTX
ELECTRO CELL
MANIPULATOR
200

Figure 14. The 0.8 mm electrode used (right), the electroporation chamber (middle), and the power supply outlet (left), used for pulsing the pollen.

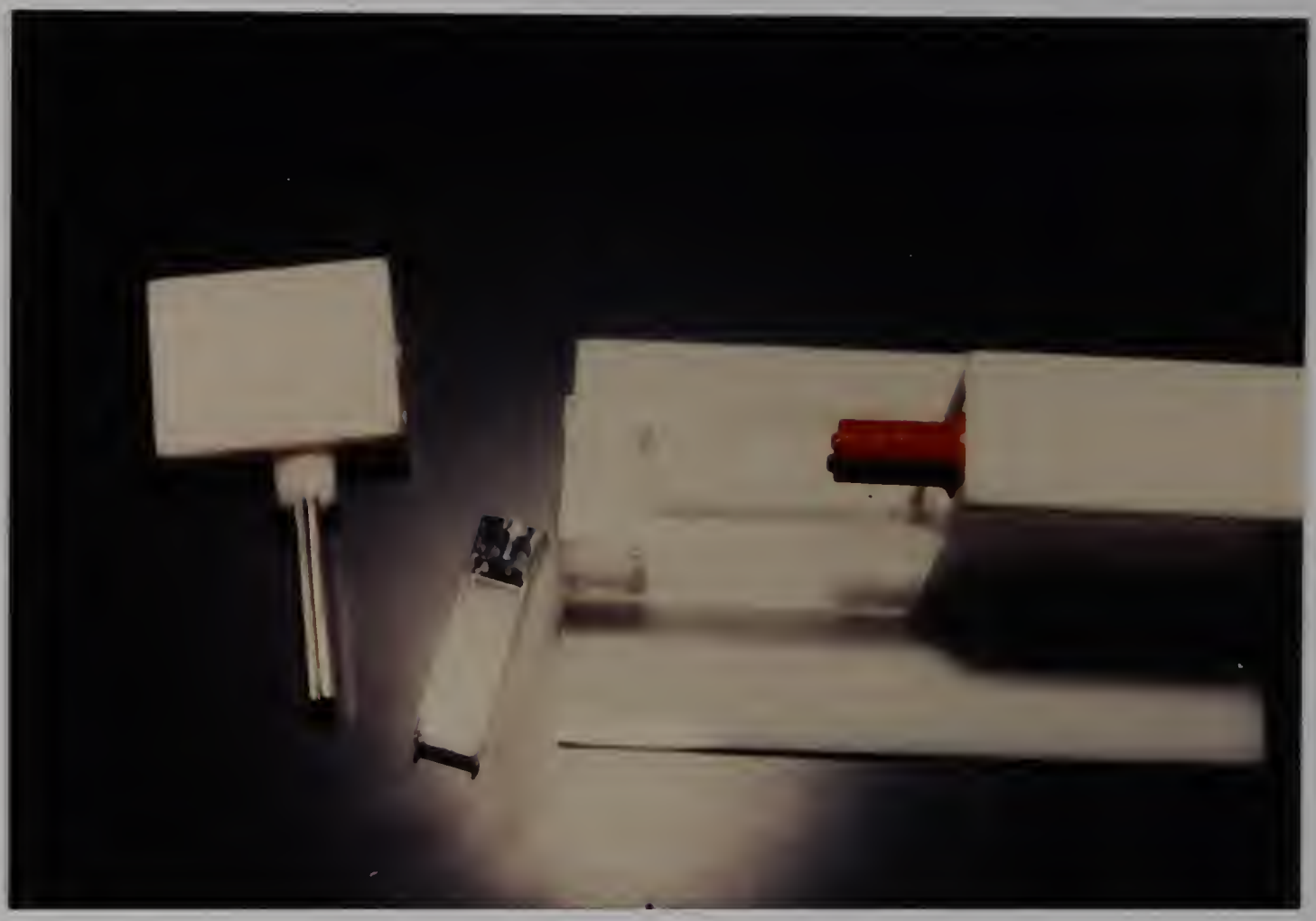

Figure 15. The effect of field strength in electroporation on lucifer yellow uptake by lily pollen.

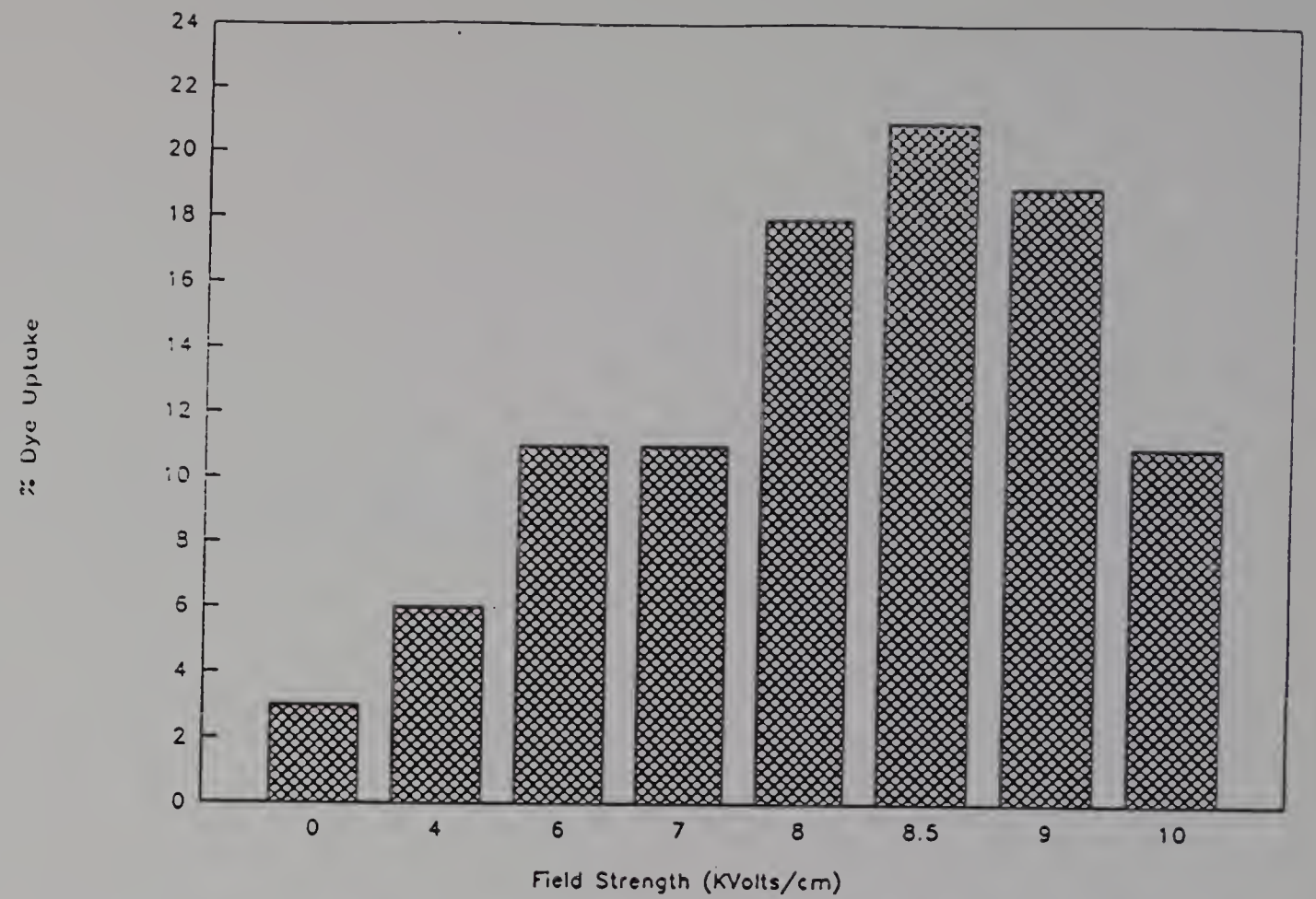
% Dye Uptake
24
22
20
18
16
14
12
10
8
6
4
2
0
0
4
6
7
8
8.5
9
10
Field Strength (KVolts/cm)

Figure 16. The effect of electroporating field strength on survival of germinating lily pollen.

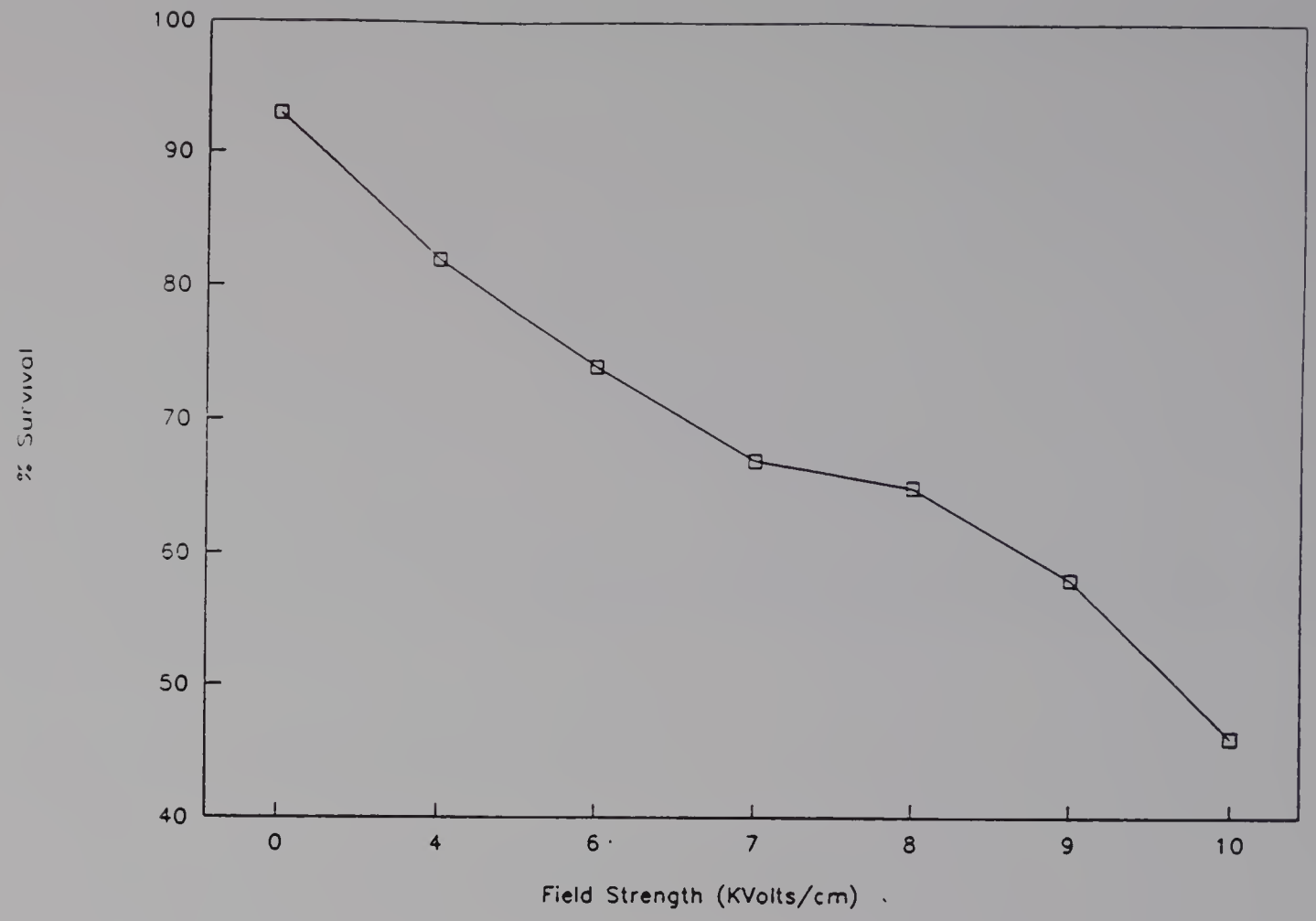
100
90
80
70
60
50
40
% Survival
0
4
6
7
8
9
10
Field Strength (KVolts/cm)

Figure 17. The effect of electroporation media on survival of germinating lily pollen.

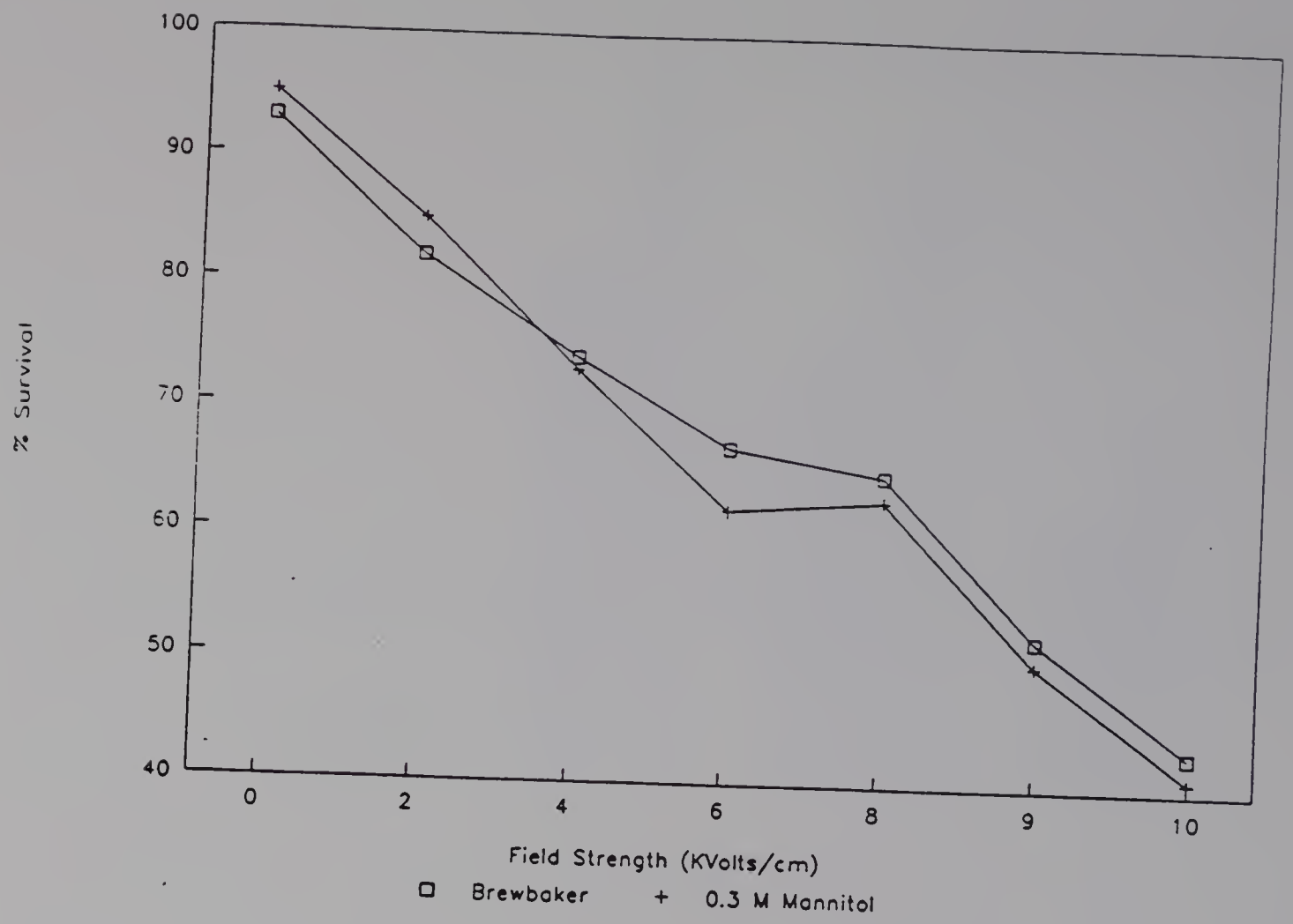
100
90
80
70
60
50
40
% Survival
0
2
4
6
8
9
10
Field Strength (KVolts/cm)
Brewbaker
0.3 M Mannitol

Figure 18. The effect of pollen concentration in the electroporating sample on lucifer yellow uptake at various field strengths.

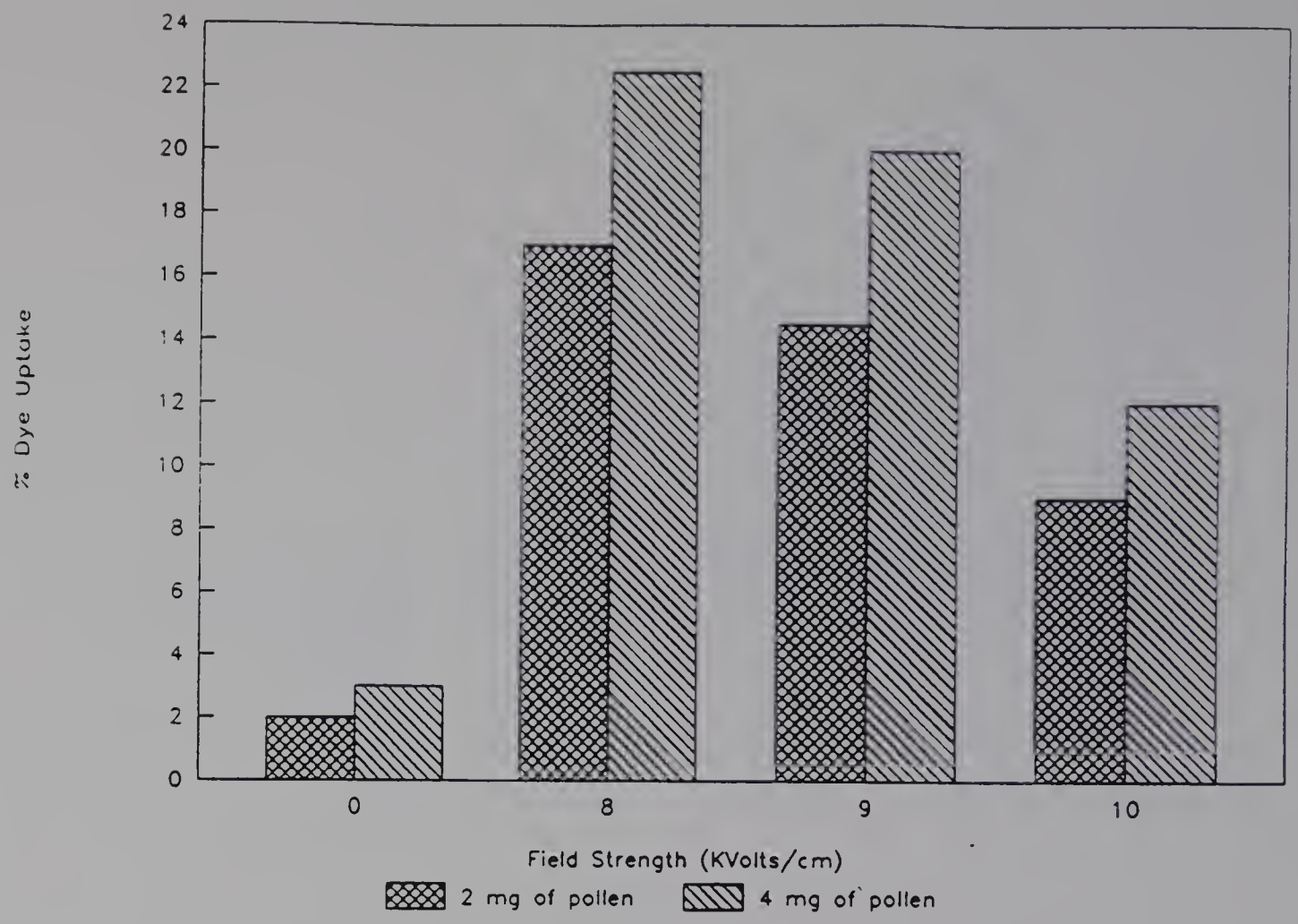
% Dye Uptake
24
22
20
18
16
14
12
10
8
6
4
2
0
0
8
9
10
Field Strength (KVolts/cm)
2 mg of pollen
4 mg of pollen

Figure 19. The effect of pollen concentration in the electroporation sample on lucifer yellow uptake.

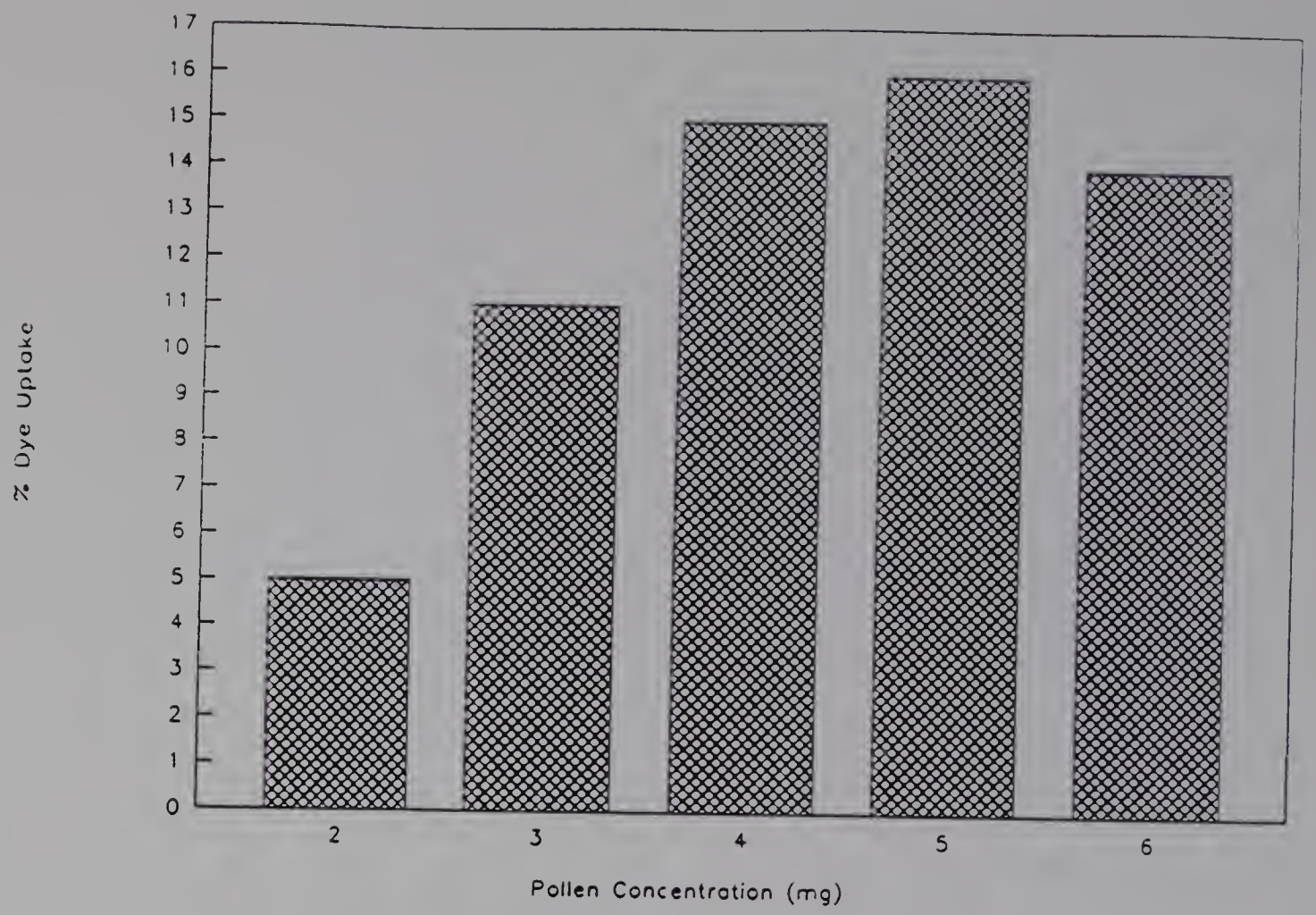
% Dye Uptake
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
2
3
4
5
6
Pollen Concentration (mg)

Figure 20. The effect of varying pulse widths with various field strengths on lucifer yellow uptake in germinating lily pollen.

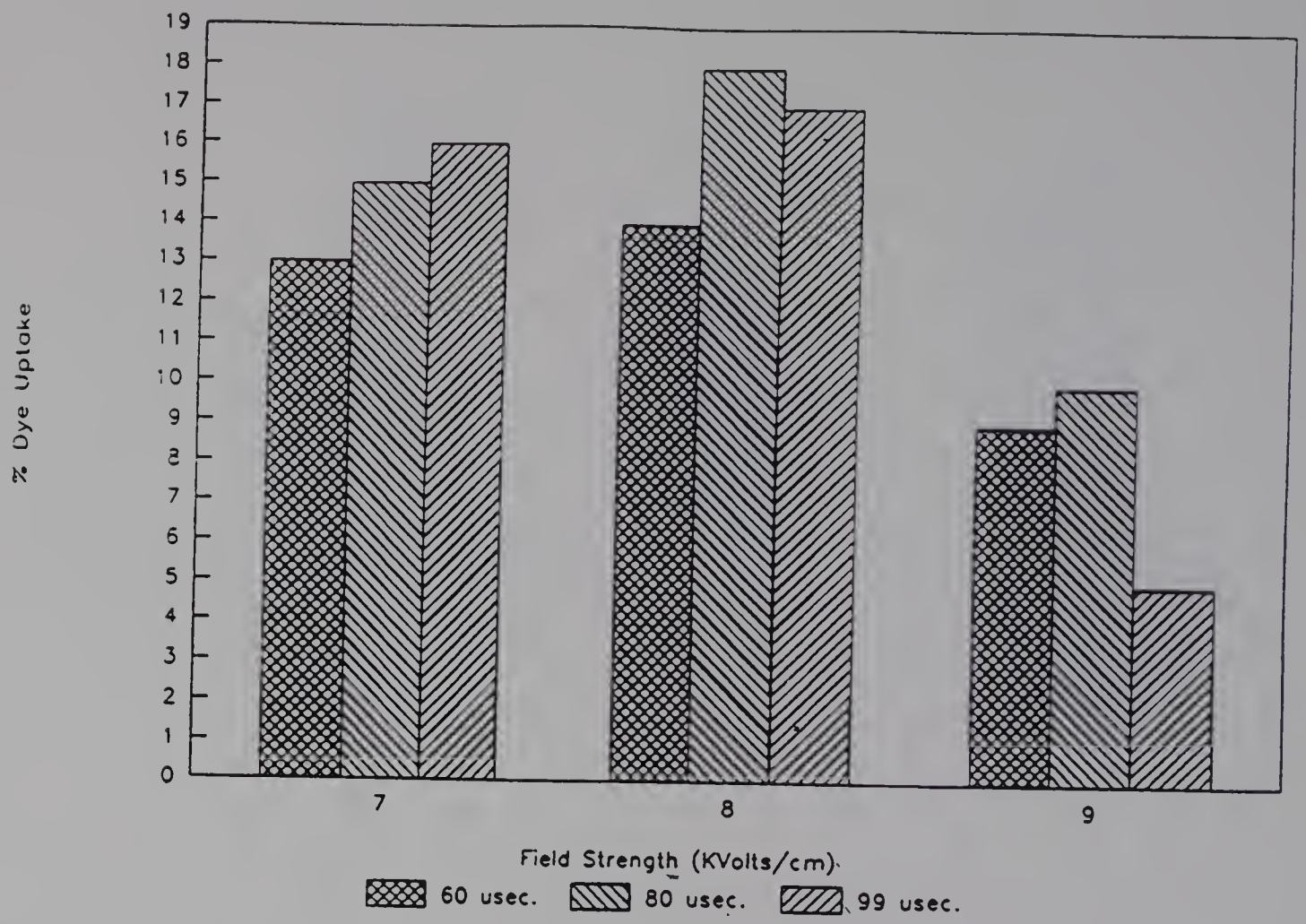
% Dye Uptake
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
7
8
9
Field Strength (KVolts/cm).
60 usec.
80 usec.
99 usec.

Figure 21. The effect of resting period after electroporation on lucifer yellow uptake in germinating lily pollen.

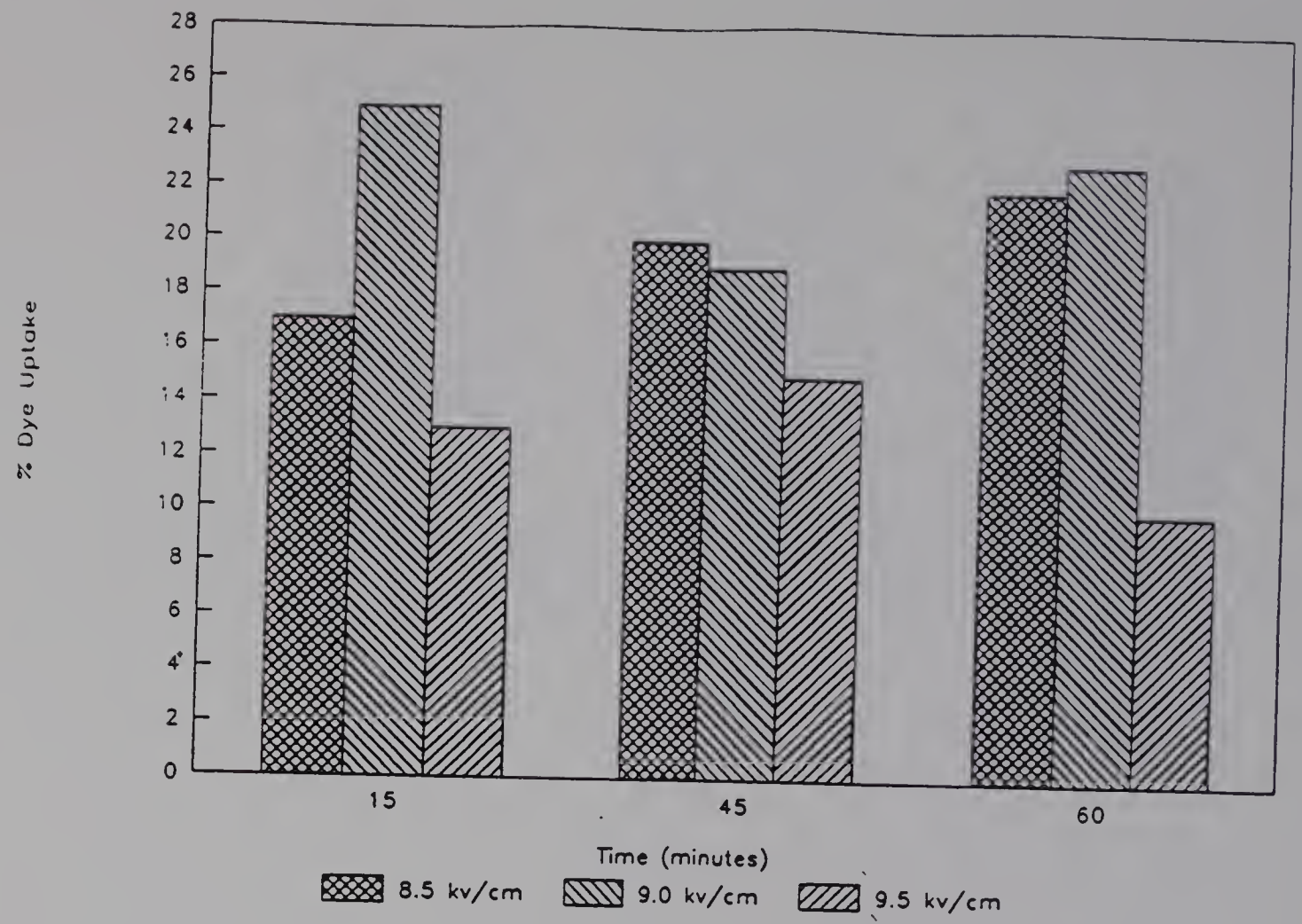
% Dye Uptake
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
15
45
60
Time (minutes)
8.5 kv/cm
9.0 kv/cm
9.5 kv/cm

Figure 22. The effect of temperature on lucifer yellow uptake in germinating lily pollen.

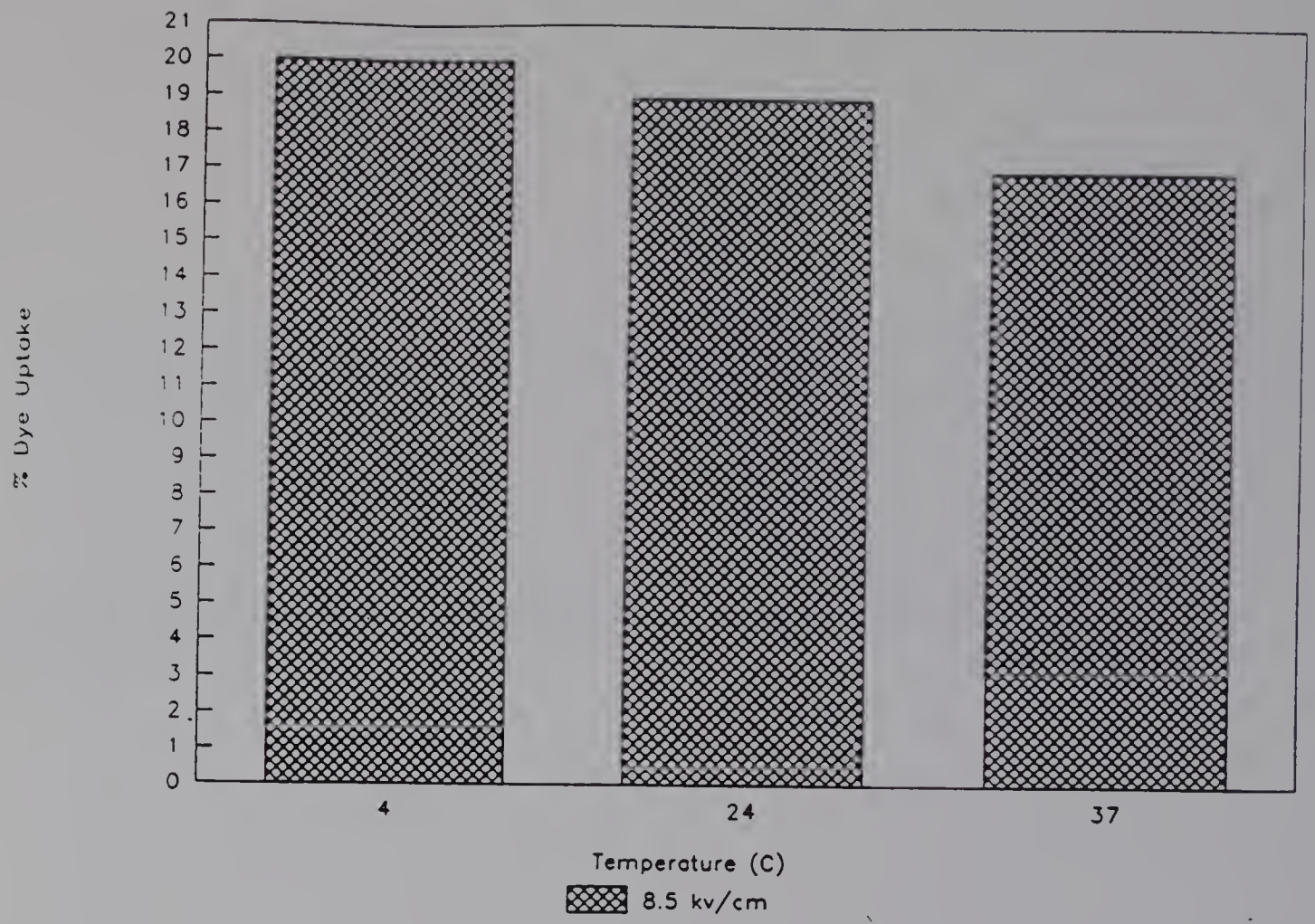
% Dye Uptake
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
4
24
37
Temperature (C)
8.5 kv/cm

Figure 23. A false positive. Control lily pollen stained blue in the histochemical assay for GUS activity.

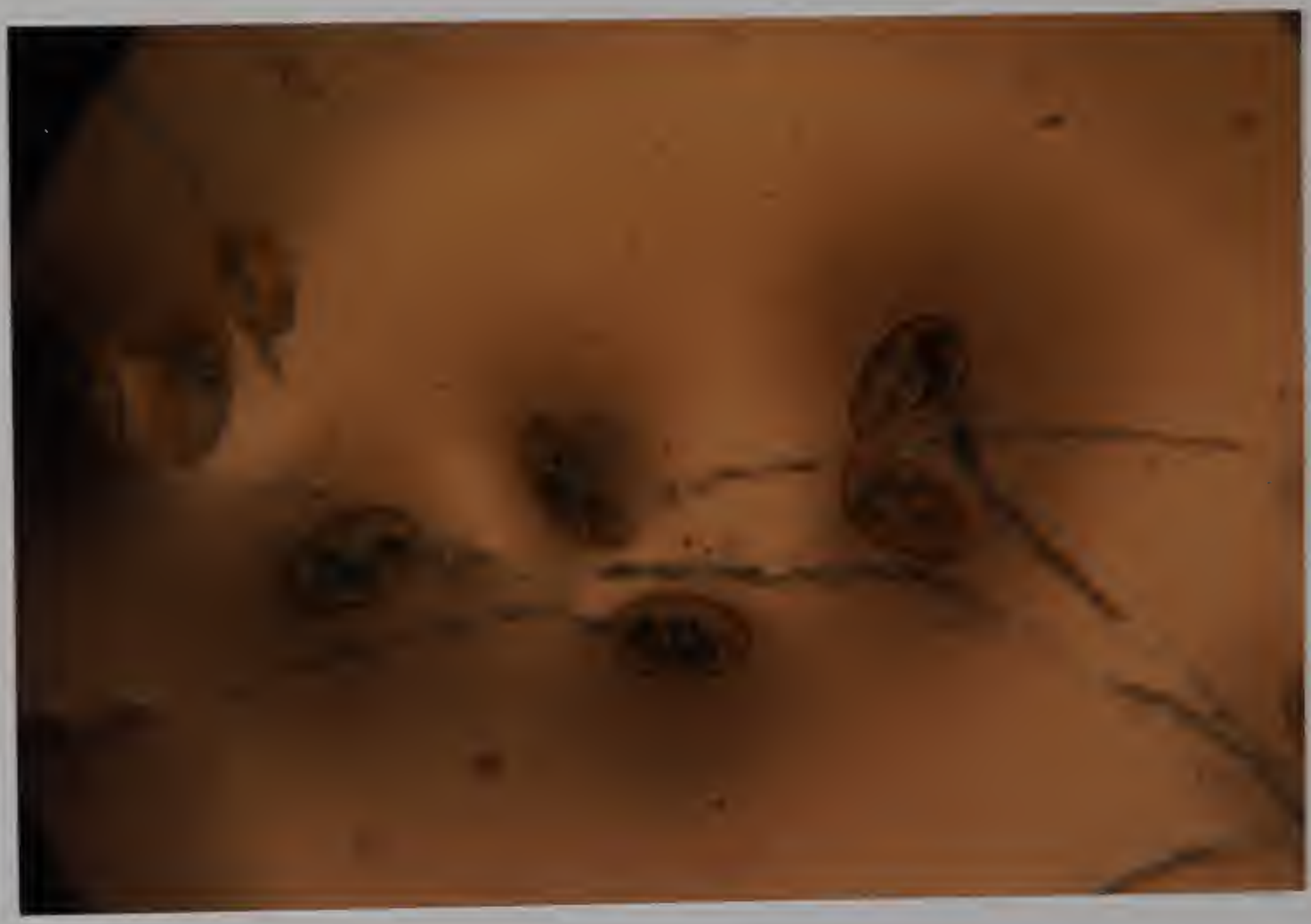

Figure 24. Lily plants at the stage for anther injection technique. The leaves on these lilies have just folded back to expose buds that are approximately 10-14 mm in length.

Figure 25. The micromanipulator used for the microinjection of lily pollen.

CHAPTER V

DISCUSSION

Most of the successful plant transformations achieved today strongly depend on Agrobacterium tumefaciens as a vector. Although there are a number of various other techniques now available these have not proven to be as competent as the Agrobacterium mediated transformations. Techniques using the microprojectile gun, PEG mediated transformants, microinjections and electroporations for plant transformations exist. The problems with many of these methods range from inefficient uptake of the foreign DNA to scrambling or breakage of the DNA to be incorporated and expressed.

All of these plant transformation methods to date require extensive tissue culture stages to achieve uptake and expression of the foreign DNA. From tissue culture the plants must then be regenerated to whole plants. In some of the high value cereal crops such as corn, wheat and soybean regeneration from protoplasts has not yet been accomplished (Joshi, 1991). Regeneration from tissue culture, also presents numerous problems such as somatic variations that can occur. Somatic variants are plants that are mutated in some way and are not identical to the parent plants. These plants may loose some of the qualities that plant breeders have selected for such as vigor or high yield. Thus in a successful plant transformation for the incorporation of a disease resistance gene, the regenerated plant may have lost other qualities that are equally or more important. Basically, tissue culturing of plants has disadvantages, both in the initial stages because it is so time consuming, and again with the possibility of somatic variants that no longer express any of the

qualities that it was selected for in the first place. Therefore, the most ideal situation would be to have a transformation technique that would surpass the tissue culturing stages.

When using pollen as the material to genetically engineer, the requirement for any tissue culture stage could be omitted. It would be more than ideal if Agrobacterium may be used as a vector, but previous experiments have indicated that this method has failed for pollen transformation. Therefore, in this study three different techniques were appraised: electroporation, microinjection of pollen, and anther injection.

Pollen has many advantages over the traditionally used plant material. The natural role of this male gametophyte is the production of two sperm cells and their transport within the pollen tube through the tissues of the style into the embryo sac in the ovule. Double fertilization then follows. The fusion of one sperm with the egg and the second sperm with the central cell results in the formation of the zygote and the primary cell, respectively (Mascarenhas 1990). If the pollen contained foreign DNA the resulting fertilization would result in genetically transformed seed. Thus genetically engineering pollen has the potential to be the most straight-forward, and most rapid form of plant transformation available today.

The technique involving injection of anther tissue has some theoretical backup but in our experiments was not found to work. There are many possibilities why this technique may have failed. The main reason for failure may be that the DNA once injected is rapidly degraded by DNases. Secondly, if the DNA actually made it inside the grain, and was not degraded, will the DNA be incorporated into the nuclear genome so that it may be expressed. Also, is this injection procedure going to affect the pollen development? The results obtained from these anther injection studies showed that, of all the buds injected approximately half of them shrivel up and die before reaching maturity, and the injected buds that make it to the

flower stage almost always show some kind of abnormality. Of the injected buds that made it to maturity, the pollen present on the injected anthers showed no sign of the foreign DNA expression. The anthers that made it to maturity, and were injected with lucifer yellow, exhibited pollen grains that were fluorescent, and very abnormal or dead . These fluorescing cells may have been the first ones injected and may have not allowed the injected material to go any further in the developing pollen. The published success in using this technique is extremely low. De la Pena et al (1987) injected young floral tillers of rye plants. Three transformed rye plants were obtained when plasmid DNA carrying a gene coding for kanamycin resistance was injected about 14 days prior to the first meiotic metaphase, into about 350 floral tillers. There have not been any other reports to my knowledge using this technique.

Another method of gene transfer is microinjection. This method of plant transformation has been successfully accomplished in tobacco mesophyll protoplasts by Crossway et al (1986). It is however, generally considered to be difficult, tedious, and is not conducive to treating large numbers of plant cells or protoplasts. Microinjection also demands a high level of skill in micromanipulation and expensive micromanipulation equipment. Microinjection as a technique, is not regularly considered when trying to achieve large scale plant transformations. It is however, widely and successfully used in animal systems for gene expression studies and indicator studies using fluorescent dyes (De Pamphilis, 1988). The microinjection of pollen has previously been utilized to study intracellular calcium gradients in germinating pollen tubes (Zhang, 1990). Microinjection of pollen for study of gene expression has not been published. This is probably due to difficulty encountered by the outer wall of the pollen grain consisting of a substance called sporopollenin which is considered one of the most resistant and toughest of all natural compounds. The presence of this substance makes injections extremely difficult. The results of this study indicate that injection of pollen tubes is possible

but are difficult and time consuming. Secondly, the injection of a pollen tube without causing severe injury or death is very difficult, so that the expression of DNA is unlikely to occur in many of the grains.

Electroporation of plants cells seems to be one of the more promising techniques. The success rate of electroporation of protoplast with plasmid DNA, as far as the number of transformants achieved, is usually low. Shilito et al. achieved uptake and expression in 2% of tobacco leaf mesophyll protoplasts using a plasmid conferring kanamycin resistance. Riggs and Bates (1986) obtained stable transformants with linearized plasmid DNA at a frequency of 2 x 10 -4/pulsed protoplasts with an optimum survival rate of 50%. However, when tobacco protoplasts were electroporated with tobacco mosaic virus (TMV) the success rate was clearly better (80 % infection), (Okada et al.) The electroporation of tobacco pollen has also been successful by Abul-Baki et al. (1990). By optimizing all of the electroporation parameters, Sauders et.al. (1989), achieved a 6 % transformation rate of the germinating tobacco pollen, while maintaining a pollen viability of 90%. The plasmid used in the transformation of tobacco pollen was pBI221, which was also used in this study.

The parameters for the electroporation of lily pollen were worked out using the highly fluorescent dye, lucifer yellow. The optimal field strength was found to be 7-9 kV/cm whereas, in the tobacco pollen study it was found to be between 8-9 kV/cm. The pulse width of 80 usec with a pollen concentration of 4mg/ 300ul was also found to be an optimal parameter. This study also indicated that other parameters such as resting period after the electroporation pulse and temperature conditions may be necessary for optimum survival and uptake. There was no expression of the foreign DNA using these parameters indicated by the lucifer yellow studies. This failure may be due to several factors. Assuming the DNA entered the pollen grain, the DNA once in the pollen grain was degraded or was not

incorporated into the genome for expression. If the DNA was incorporated but was not recognized by the RNA polymerases it would also not be expressed. Or maybe the DNA was transcribed and translated but not detected by the most sensitive assay for the reporter gene available. These are only some of the obvious possibilities.

The DNA that was used mostly in these studies was the plasmid pBI221. The plasmid was designed, and thus suggested in the instruction catalog to be used ideally in direct gene transfer systems, such as electroporation and microinjection. This plasmid was the smallest plasmid available that contained both a known constitutive promoter, a reporter gene, and a selective antibiotic marker. This plasmid was thought to have the best chance for obtaining expression mainly do to its size and the fact that the GUS cassette was driven by the 35s CaMV promoter, which was shown to be read and expressed at high levels in most plants when incorporated into the genome.

The plasmid pBI121 was the largest plasmid used (13kb) which was more than double the size of pBI221. The plasmid pBI121 was designed mainly for the use of plant transformation studies involving *A. tumefaciens* as the vector , due to the presence of the Ti DNA right and left border sequences contained on the plasmid. The large size of this plasmid will limit its application in direct gene transfer techniques.

The plasmid Zm13 seemed initially a very desirable plasmid to use in these studies due to its small size, the presence of the reporter gene GUS, and it contained a pollen specific promoter isolated from a monocot (Hanson, 1989). However there were problems in using this plasmid. The first being that the plasmid did not contain a selectable marker that could be expressed in plant cells. Secondly, the pollen specific promoter meant that the expression of the promoter was only expressed at the pollen stage; therefore, no expression would be expected in the sporophyte

tissue. This was thus an unreasonable plasmid to use for studies other than trying to just transform the pollen.

The plasmid pH1S has a small size, and a fungal promoter driving the expression of the selectable marker hyg B gene, which encodes for hygromycin B phosphotransferase. There are however numerous problems in using this plasmid. First, the fungal promoter isolated from *Cochliobolus_heterostrophus* has not been used in plant transformation studies before and thus, it is not known whether this promoter will be recognized and read in a plant system. Secondly, there is no reporter gene on this plasmid. A reporter gene is essential when initially experimenting with a technique, to see if expression of the foreign DNA may be achieved. This plasmid, because it only carries a selectable marker, may only be screened at the pollen and or the next generation seed stage depending on which method of transformation is being used.

In comparing the techniques used in this study, the most reasonable one would seem to be electroporation as the most efficient for large scale plant transformation studies. Microinjection of pollen is ruled out due to the inability to inject large amounts of pollen grains successfully. Microinjection of pollen is more suited to studies where one successful injection is sufficient for the experiment at hand. The injection of anthers/meiocytes at the premeiotic stage seems like the best situation but only if the incorporation can some how be improved along with the survival rate of the injected buds. The injection of these meiocytes would be the most ideal situation for pollen transformation. The injected meiocytes would develop to the mature pollen stage. This pollen would then be screened with the appropriate antibiotic that is contained on the plasmid. The pollen would be germinated in the presence of the appropriate anitibiotic right from the start of the germination process. The pollen not expressing the foreign DNA would not be able to germinate. On the other hand, the pollen expressing resistance would be able to

successfully germinate. This germinating pollen would then be placed in a receptive stigma so that fertilization would occur. The resulting seed would not need to be screened again, but such a screen would remain an option. Once new plants are formed they may be tested for the expression of the reporter gene.

If the techniques of microinjection and electroporation were used, no screening would be done initially at the pollen stage, but a GUS assay of 1/10 of the total pollen sample would be sacrificed to test if the transformation procedure was successful. The seed screening would then be a crucial event. Then, the rest of the procedure would proceed as explained above with the meiocyte injection technique.

Future experiments involving pollen transformation should be investigated. Problems addressing efficiency should be worked out first, using well known/easily transformed plants such as tobacco or arabidopsis. Other techniques involving the pollen tube pathway should also be investigated.

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