

## Research Article

### ***In vitro* Manipulation of *Impatiens glandulifera* Pollen for Transporting Extracellular Substances to the Embryo Sac**

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**Abstract:** Pollen from *Impatiens glandulifera* were manipulated *in vitro* to investigate the possibility of using them 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 and loading of fluorescent probes by plasmolysis/endocytosis via germinating pollen.

**Keywords:** Endocytosis, fluorescent probes, plasmolysis, pollen

## INTRODUCTION

The potential scientific rewards from *in vitro* manipulation of plant gametes, as compared to *in vivo* observation, are enormous. Practical, commercial benefits might also be considerable. *In vitro* fertilization might provide a means of circumventing incompatibility mechanisms that operate within the stigma and style. This in turn would allow inter-specific crosses to be made, broadening the genetic base of crop plants.

Recent evidence has initiated that a large proportion of the micro gametophyte genome is transcribed and translated during pollen development and also expressed during the sporophytic stage of the life cycle. It was reported that at least 64% of the pollen mRNA population of *Tradescantia paludosa* and *Zea mays* was also expressed in shoot tissue (Tanksley *et al.*, 1981). 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 fertilization and the role of sperm cells in this process. Various techniques have been developed to obtain isolated sperm cells (Russel, 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, was conducted by the soaking of pollen with various nucleic acid preparations (De Wet *et al.*, 1984). They have suggested that germinating pollen tubes can take up exogenous DNA. It was reported that the absorption of the DNA through the pollen tube of maize (*Zea mays* L.) and its incorporation into the genome of the zygote during fertilization (De Wet *et al.*, 1985).

By contrast with animals, fertilization 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.

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

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

The objective of this research is to introduce xenobiotics through plasmolysis/deplasmolysis of pollen and assess their fate during the fertilization process as an attempt to assess the feasibility of introducing foreign DNA that would allow the control and manipulation of the chemical and physical environment for gamete and developing zygote.

## MATERIALS AND METHODS

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 sterilized by 70% ethanol for 1 min, 10% sodium hypochlorite for 10 min and washed thoroughly in sterilized 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 (Mumford, 1988). The germinated seeds were then transplanted in compost 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 14 h/day.

The chemicals used included Murishige and Skoog (MS) basal medium (Murishige and Skoog, 1962), fluorescein diacetate, Manitol, Lucifer Yellow carbohydrazide, 4',6-Diamidino-2-Phenylindole (DAPI) are supplied by Sigma Chemical Co., St. Louis, USA. Boric acid, Acridine orange, Aniline blue, supplied by BDH Chemicals Ltd., Poole, England. Calcofluor white M2R new is supplied by Polysciences Inc., Warrington, PA, USA.

Three culture techniques were applied: sitting-drop, hanging-drop and cellophane sheets. 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 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. Pollen growth was assessed under different temperature (4, 8°C and room temperature) and different humidity levels (0, 55.0, 75.0 and 92.5 RM, respectively).

Pollen viability testing was carried out using Fluorescein Diacetate (FDA), Calcofluor white M2R new (CFW). 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 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 fluorescein 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. Fluorescein 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.

The pollen developmental state of *I. glandulifera* was assessed floral buds from different growth stages from the initial stage up to the stage where the anther split open. Buds were treated by Feulgen staining and 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. In order to monitor the behavior of pollen nuclei *in vitro*, pollen was cultured in the basal medium described earlier with the addition of 0.01% mg/mL 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 by two Nikon microscopes. The DIAPHOT-TMD, with a 35 mm 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.

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 plasmolyzing pollen in the presence of fluorescent probe (LY-CH) and deplasmolyzing it. Experiments were conducted to trace the probes into the pollen. Three batches of fresh pollen were compared; a control batch, plasmolysed and deplasmolyzed in mannitol and plasmolysed and deplasmolyzed in the fluorescent probe. These batches were fixed, dehydrated and imbedded 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 *in vitro*.

In an attempt to detect whether *in vitro* pollination was effective and whether plasmolysed/deplasmolyzed pollen could reach ovules, after pollination with control and plasmolysed/deplasmolyzed pollen, ovules were dissected and stained with a mixed stain of 1% w/v aniline blue and the fluorescent brightener 'CFW New', after pollination with control and plasmolysed/deplasmolyzed pollen.

## RESULTS AND DISCUSSION

The results revealed non-significant differences between the three culture techniques. Longer pollen tubes were obtained with 5% (w/v) sucrose (258.81 µm) and a decreasing trend with the increase in sucrose concentration from 10-20% (w/v) (203.41 and 23.04

µm, respectively). Boric acid, on the other hand, gave longer pollen tubes (168.97 µm) 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. When both sucrose (5% (w/v)) and boric acid (0.005-0.02%) (w/v) were combined, pollen tubes up to 552 µm long were obtained; longer than were obtained using the medium in which both nutrients were used separately. The addition of 100 ppm of Mg, Ca and K, however, showed significant variation in the role played by both sucrose and boric acid, particularly with potassium. According to these results, the best medium for the culture of *I. glandulifera* pollen which provided longer pollen tubes as 5% (w/v) sucrose, 0.01% (w/v) boric acid supplemented by 100 ppm potassium nitrate and 100 ppm magnesium sulphate. This medium was used throughout succeeding experiments. Pollen tube growth was significantly better at 8°C than at room temperature; however they also grew under lower temperatures down to 4°C. Despite the fact that pollen grew better under room temperature they showed a higher rate of bursting compared to lower temperature (4-8°C) under which no pollen bursting was observed.

Both concentrations of CFW (0.1 and 1.0% w/v) gave similar effects on fluorescence intensity. CFW was found to be less toxic up to 1.0% (w/v) as pollen readily germinated in a medium containing CFW at this concentration. Therefore a concentration of 0.1% (w/v) 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 and 8°C) compared to room temperature; however, at high temperature (60°C) high bursting and no germination resulted, which agrees with other reports in which pollen bursting at temperatures higher than 35°C was reported (Vasil and Bose, 1959). Treatment of pollen tubes with 1% (w/v) CFW for 5 min showed a significant variation in fluorescence intensity between pollen incubated at different temperature for 24 h. 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. The reaction of the FCR test by *I. glandulifera* 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 results.

The effect of plasmolysis on pollen tubes was non-significant on the final length obtained after subsequent rehydration and growth. Pollen loaded with LY-CH after plasmolysis in the presence of the probe and deplasmolysis. Pollen nuclei fluoresced bright yellow under blue excitation, due to the accumulation of

vesicles around nuclei. Uptake of fluorescein by pollen was evident after plasmolysis and deplasmolysis. Strong fluorescence resulted under blue filter. 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.

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

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 medium developed so far are mainly comprised of sucrose, boric acid and other nutrients (e.g., Ca, K, Mg). Longer pollen tubes were obtained from *I. glandulifera* pollen when sucrose and boric acid were combined in the medium. This finding is in line with those who postulated that borate ions react with sugar molecules to form an ionizable sugar borate complex which moves through the cellular membranes more readily than non-ionized sugar molecules. The variation in response to other nutrient elements may be species-specific.

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 pre-incubated 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 indication that the FCR test shown only an approximate estimate of pollen germinability. Pollen germination percentages lower than FCR were reported (LaPorta 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, does not precisely reflect the actual viability status of the pollen grains. To show how the CFW test works with

*I. glandulifera*, we refer to studies which used CFW to distinguish between living and dead cells from a variety of animal and plant species (Fischer *et al.*, 1985). 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. The behavior of survival of pollen are influenced by both environment and genotype. Pollen viability varies with the nutrition of the parent plant. It also requires reconditioning and post-maturation development. Some viability testing methods may give an unrealistic estimate of quality (Heslop-Harrison, 1992). The use of decolorized aniline blue has proved to be an effective technique that permits rapid localization and visualization 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 some studies (Vasil, 1987). However, even the shape of callose plugs varies in pollen tubes growing under similar conditions in vivo and in vitro (Hussein, 1993).

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 that plant cells can take up the fluorescent dye and deposit it in the vacuole (Hilmer *et al.*, 1989). The results shown in this study provide new evidence of uptake of LY-CH by pollen after plasmolysis and deplasmolysis. Microtome sections have clearly shown the accumulation of LY-CH around the pollen nuclei, which agrees with findings on vesicles trapped in the thin layer of the cytoplasm surrounding the nucleus during the rapid plasmolysis of onion epidermal cells (Oparka *et al.*, 1991). As the probed 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 by pollen was through endocytosis.

Of all the vesicle trafficking systems, the process of receptor-mediated endocytosis is considered as the best characterized (Hawes *et al.*, 1991). It was reported that the endocytic pathway involves endocytosis by coated pits, delivery to coated vesicles, smooth vesicles, the partially coated reticulum and Golgi, multi-vesicular 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.

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 membrane-bound vesicles could possibly fuse together,

fuse with internal membrane, transported across the wall, or may break down and discharge probe into cells.

The various aspects of this 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. Such a significant finding would open the door for manipulation of pollen through plasmolysis/deplasmolysis 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 goal.

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