

Manipulation of Ovaries/Ovules and Clearance and Isolation of Embryo Sacs of *Impatiens glandulifera* and *Nicotiana tabacum*

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Abstract: The objective of this research was to develop techniques to clear and/or isolate embryo sacs of *Impatiens glandulifera* and *Nicotiana tabacum*, to facilitate microscopic examination for subsequent experimentation on *in vitro* fertilization of flowering plants. Two techniques were applied: The clearing and enzymic maceration. Both of the 2 adopted techniques were effective in revealing embryo sacs of the studied plant species. A fixative prepared from propionic acid, formaldehyde, ethanol (50%) in the ratio 5:5:90 was used. The clearing solution was prepared from lactic acid (85%), chloral hydrate, phenol, clove oil and xylene in the ratio 2:2:2:2:1 by weight. Both the clearing and the enzymic maceration techniques proved effective in revealing embryo sacs of *I. glandulifera* and *N. tabacum*.

Keywords: Embryo sac, enzymic maceration, fixation techniques, ovules

INTRODUCTION

Sexuality was described by Fryxell (1957) as conferring on plants a "strong selective advantage" which provides the opportunity for genetic recombination and thus variations in populations. This in turn confers evolutionary plasticity in the face of changing environmental conditions. The control of sexual reproduction and thus recombination, has long been a major objective for experimental botanists and plant breeders.

The understanding of early zygote development in animals has advanced rapidly, as a direct result of the fact that *in vitro* fertilization of female gametes can be achieved with relative ease. In plants, the fertilization event takes place deep in the ovule, inside the protective carpel. Female ovules are retained within numerous surrounding sporophytic cells throughout maturation, sexual function and germination. This has prevented all but the most rudimentary physiological characterization of the gametophytic cells that produce the functional female germ unit (Dumas *et al.*, 1984) because of this in-accessibility the precise details of the fertilization mechanism and early embryo development have still to be described.

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

and physical environment for gamete and developing zygote. Practical, commercial benefits might also be considerable. *In vitro* fertilization might provide a means of circumventing incompatibility mechanisms that operate within the stigma and style. This in turn would allow new inter-specific crosses to be made, broadening the genetic base of crop plants.

Recent evidence has indicated 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 (Tanksley *et al.*, 1981; Willing and Mascarenhas, 1984). It was reported that at least 64% of the pollen mRNA population of *Tradescantia paludosa* and *Zea mays* was also expressed in shoot tissue (Willing and Mascarenhas, 1984). A similar percentage of genetic overlap between sporophytic and gametophytic phases was also reported in *Lycopersicon esculentum* and *Zea mays* (Tanksley *et al.*, 1981; Sari Gorla *et al.*, 1986).

Isolation of egg cells has been reported in maize (Kranz *et al.*, 1995), wheat (Kovacs *et al.*, 1994), rye grass (Van Der Mass *et al.*, 1993), Brassica (Kato *et al.*, 1997), Plumbago (Huang and Russell, 1989) and tobacco (Tian and Russell, 1997). Manual isolation of embryo sacs was conducted by other studies (Lakshmi *et al.*, 2002). Based on the histological observations of the ovule and embryo sac of *Alstroemeria*, an attempt was made to isolate the female gamete (egg cell) by enzyme maceration in combination with micro-dissection (Hoshino *et al.*, 2006). However, because of the difficulty to isolate the embryo sacs manually at some stage, further identification of features conducive to manual dissection is yet to be undertaken.

The aim of this research is to develop techniques to clear and/or isolate embryo sacs in an attempt to facilitate examining them microscopically for traces of fluorescent probes loaded via germinating pollen or via the vascular system. These included enzymic maceration, tissue clearing solutions and fixation, embedding and serial sectioning.

MATERIALS AND METHODS

Many techniques have been developed for the study of significant features in the embryology of angiosperms, specifically ovule development, megasporogenesis and megagametogenesis. Each technique, however, reveals specific information and serves certain objectives, which could not be accomplished using other techniques. Nevertheless, some techniques have been devised for particular species. This study was partially conducted at Durham University laboratories (UK) as part of a Ph.D programme and finalized at Al Jouf University (KSA) during the period 2011-2012. Two techniques were employed on fresh and fixed material from *I. glandulifera* and *N. tabacum* in an attempt to derive a convenient method. These include the clearing and the enzymic maceration techniques.

Clearance of embryo sacs by fixing and clearing solutions: The fixative for both *I. glandulifera* and *N. tabacum* ovules was prepared from propionic acid, formaldehyde, ethanol (50%) in the ratio 5:5:90. The clearing solution was prepared from lactic acid (85%), chloral hydrate, phenol, clove oil and xylene in the ratio 2:2:2:2:1 by weight. Plant ovaries were fixed for 24 h and then immersed in the clearing solution for 24 h at room temperature (24±1°C). Ovules were dissected under a stereomicroscope and transferred with a small amount of clearing fluid to slides and examined using a Nikon Optiphot-2 microscope. Pistils from both species were collected from floral buds at the time of anther opening and 1 week after pollinating *in vivo*. Dissected ovules fixed for 24 h were immersed in the clearing solution for 24 h and examined microscopically by Differential Interference Contrast (DIC) microscopy using a Nikon Optiphot-2 Microscope.

Isolation of embryo sacs by enzymic maceration: Ovules from *I. glandulifera* were excised and macerated enzymically using 2% (w/v) driselase solution for 2-3 h at 24-26°C. The enzyme solution was prepared from 2% driselase, 0.65 M mannitol and 0.25% potassium dextran sulphate. Ovules were incubated in 1 mL of enzyme solution and placed in a micro-shaker for 6 h. After incubation, plant material was centrifuged for 5 min (1500 rpm) and the pellet was washed 3 times using a washing solution of 0.65 M mannitol and examined under the microscope. When

isolation had occurred, the suspension was centrifuged at 1,500 rpm for 5 min. The supernatant was discarded and the precipitate washed three times by resuspension and recentrifugation with 0.1 M sucrose solution. The isolated embryo sacs were examined for viability using the FDA test.

RESULTS AND DISCUSSION

Both the clearing and the enzymic maceration techniques proved effective in revealing embryo sacs of *I. glandulifera* as indicated in Fig. 1 and 2, respectively. The embryo is not clearly visible by these methods (Fig. 1a), 7 days after pollination *in vitro*. Isolated embryo sacs of *I. glandulifera* were tested by the Fluorocein Diacetate Test and they showed no sign of viability as indicated by their negative fluorochromatic reaction. The enzymic maceration technique revealed isolated embryo sacs (Fig. 2) with intact embryo sac walls, particularly at the chalazal and micropylar zones. This finding agrees with that of Zhou and Yang (1985) whose results provided evidence of the presence of cutin in the snapdragon embryo sac wall, which may be the reason why ovular tissues macerated enzymically remain intact, the cutin being resistant to the enzyme action. On the other hand, the response of *N. tabacum* to the clearing technique was much better than that of *I. glandulifera* in terms of the clarity of the embryo sac elements (Fig. 3). DIC photographs clearly showed embryo sac elements such as the egg cell, the central cell with polar nuclei and central vacuole, the synergids and antipodal cells.

The structure of embryo sac elements observed in this study agrees with the results obtained by Shi-Yi *et al.* (1985) who used a prolonged 8 h enzymic maceration technique. The egg cell is smaller than that of the synergid and the antipodal cells were smaller than the egg. But the central cell was the largest and possessed 2 polar nuclei and a large central vacuole.

The embryo sac clearing and enzymic maceration techniques adopted in this study were successful in the clearing of embryo sac elements as well as in the isolating of intact embryo sacs of *I. glandulifera* and *N. tabacum*. However, the embryo sacs isolated by enzymic maceration of *I. glandulifera* ovules were not viable as expressed by their negative fluorochromatic reaction, most probably because of the enzymic degradation of the cellulose in the wall during isolation. The reliability of such techniques often varies from plant to plant according to ovary and ovule size and structural complexity. The fact that the embryo sac is deeply buried in the ovule tissues makes it beyond the reach of biologists, compared with its male counterpart, the pollen, unless it can be isolated. In related studies, the isolation of viable egg cells has been achieved on wheat without enzymic maceration of the ovules. Two, 4-D applied to the stigmas resulted 3 to 7 days

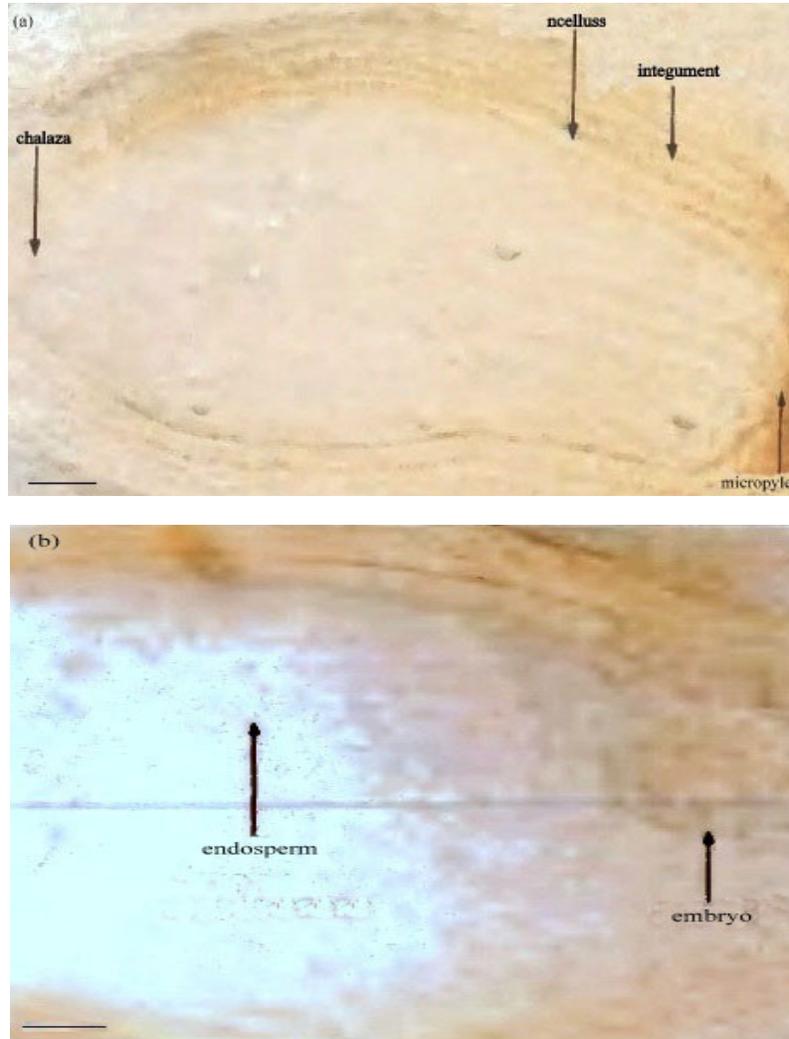


Fig. 1: Clearance of embryo sacs of *Impatiens glandulifera* ovules, (a) at anther opening and, (b) 7 days post-pollinating *in vivo*, using fixing and clearing solutions (Bars = 50 μ m)

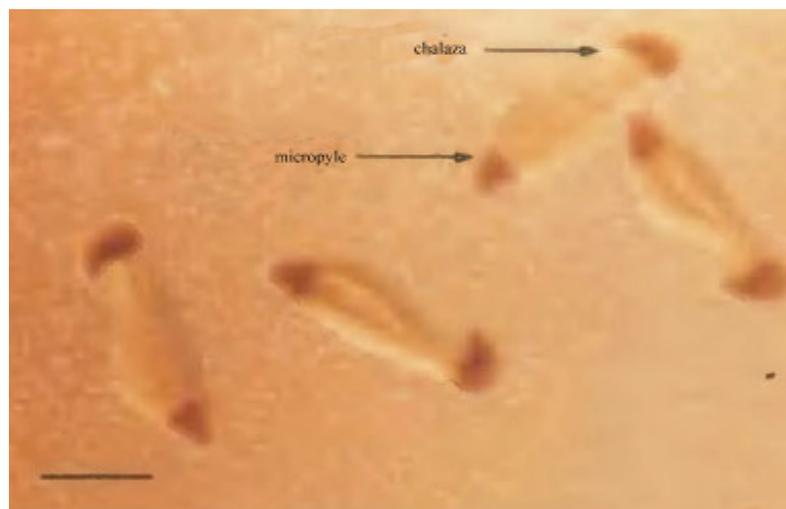
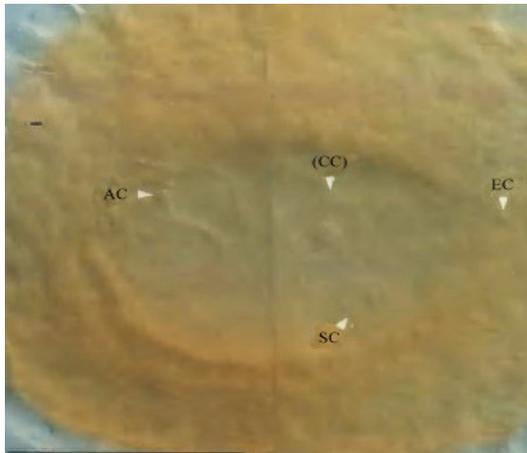


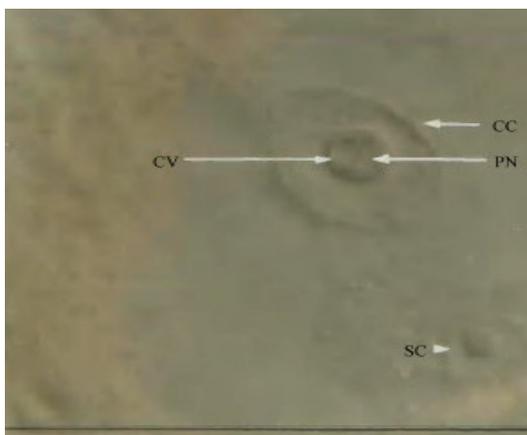
Fig. 2: Enzymic maceration of *Impatiens glandulifera* embryo sacs after incubation of ovaries in enzymic solution for 4 h (Bars = 50 μ m)



(a)



(b)



(c)

Fig. 3: Clearance of embryo sacs from *Nicotiana tabacum* ovules at anther opening, using fixing and clearing solutions. Embryo sac elements shown are the Egg Cell (EC), Central Cell (CC) with two Polar Nuclei (PN) and a Central Vacuole (CV), Synergid Cells (SC) and two Antipodal Cells (AC) (Bars = 50 μ m)

later in soft ovule tissues which disintegrated upon mechanical manipulation (Miklós *et al.*, 1994)

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

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

The embryo sac is much more difficult to manipulate than pollen due to its inaccessibility. Each of the three techniques used in clearing embryo sacs reveals some information and the use of all the 3 techniques is important if a clear picture is to be drawn.

It can be concluded that the use of the fixing medium and clearing technique described in the methods section has proved satisfactory in clearing embryo sac in *I. glandulifera* and *N. tabacum*. However, not all elements could be seen simultaneously as DIC optics views 1 optical plane at a time. Furthermore, the fixing and clearing technique affected the fluorescent probes loaded and must therefore be

considered unsatisfactory methods for detecting these probes in the embryo sac. On the other hand, the fixing, dehydrating and embedding of plant ovules in LR White can be considered a satisfactory technique in assessing uptake of fluorescent probes into embryo sacs. This is evident from the traces of LY-CH detected in *I. glandulifera* embryo sac after loading of the probe via the pedicel (Hussein, 1994).

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

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