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# NEW TECHNIQUES AND RECENT FINDINGS IN EMBRYOLOGICAL RESEARCHES (**)

**Riassunto** — *Nuove tecniche e recenti acquisizioni nelle ricerche di embriologia vegetale.* In questa breve minirassegna sono riportate sia le tecniche più recenti, in uso nelle ricerche embriologiche, che le più recenti acquisizioni di questa disciplina. Il testo è ricapitolato in due tabelle in cui sono schematicamente enumerate sia le più recenti tecniche, con gli scopi cui servono, che le recenti acquisizioni, con i metodi con cui sono state fatte.

**Abstract** — This mini-review reports and comments either new techniques and recent findings in the embryological researches. The text is summarized in two tables where the new techniques and their utility and the main discoveries with the relative methods by which they where made, are reported.

**Key words** — Embryology, cytological techniques.

## INTRODUCTION

The development of scientific knowledge is often determined by fundamental factors which occur in succession. The perfecting of new instruments and thus new methods leads to new avenues of research. This is followed by the synthesis of the findings by noteworthy scientists and the formulation of theories which in turn often lead to the improvement of instrumentation and the cycle recommences.

When Leeuwenhoek invented the microscope in the second half of the seventeenth century, research began on small organisms and

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the organization of larger ones. Many disciplines originated and many microorganisms were identified, but it was not until the microscope was perfected by Amici at the beginning of the nineteenth century that Biology took a real step forward. Incidentally, Amici was not a biologist but a mathematical physicist and his discoveries in the field of biology did not arise out of a desire to enter a new field, or a hobby, but a wish to perfect his instruments through the observation of very fine structures. In this field Amici anticipated the modern interdisciplinary sciences and the manager-scientist of today who exploits his discoveries. He owned a workshop which produced microscopes which he sold to his biologist colleagues.

The purpose of this mini-review is to outline the new techniques in embryological research in use since the sixties, and the new knowledge they have brought. The text is summarized in two Tables: the first lists the new techniques and their utility; the second lists the main discoveries in embryology with the relative new methods by which they were made.

## Recent techniques

The transmission electron microscope (TEM) was developed in the forties and since the sixties technical improvements in the instruments and samples preparation techniques have been enormous. There are now many fixation and embedding techniques and even histochemistry, which until a few years ago was exclusively the domain of the optical microscope, is making remarkable progress. In embryology where the spatial configuration of certain structures is of particular importance, it has become common to use serial sections to reconstruct cells or parts thereof with the aid of a computer (Moens & Moens, 1981). Embryological research is often frustrating because it is difficult to find a particular phase of development or to orientate the plane of section. To obviate these difficulties several new techniques have recently been perfected, according to which 5-10 μ sections are made, examined to see if they are relevant and the chosen sections then embedded (Wilms, 1980a). These techniques are particularly suited to the study of the development of the female gametophyte and of the embryo-suspensor after fertilization.

The scanning electron microscope (SEM) began to develop at the beginning of the seventies. It is particularly useful for morphological

TAB. 1 - *Recent techniques and their purposes in embryological researches.*

| TECHNIQUES | | | n° | PURPOSES |
|---|---|---|---|---|
| ELECTRON MICROSCOPY | TEM | re-embedding | 1 | select the useful stage |
| | | serial sections → computer | 2 | tridimensional reconstruction |
| | | histochemistry | 3 | localization of molecules and polymers |
| | SEM | external morphology | 4 | taxonomy |
| | | after sectioning fresh or fixed material | 5 | tridimensional structures of cells and/or tissues |
| | | after removal of embedding medium | 6 | |
| | | after freeze fracture and cytoplasmic maceration | 7 | |
| AUTORADIOGRAPHY | | | 8 | follow the pathway and/or the localization of some labeled molecules |
| INTERFERENCE CONTRAST MICROSCOPY | | | 9 | morphology without staining and/or fixation |
| FLUORESCENCE MICROSCOPY | | autoflorescence | 10 | evidentiate some molecules |
| | | fluorochromes | 11 | histochemistry |
| | | fluorescent antibodies | 12 | immunohistochemistry |

TAB. 2 - *Recent topics in embryological research and the techniques used.*

| | | used techniques (*) |
|---|---|---|
| OVULE | polarity during megasporogenesis | 3, 10 |
| | nucleoloids | 3, 8 |
| | integumentary tapetum | 3, 8 |
| | embryosac organization and fertilization | 1, 2, 3 |
| ANTHER | pollen walls origin and development | 3, 7, 10, 11 |
| | pollen walls proteins and allergenes | 3, 12, 13 |
| | nucleoloids | 3, 8 |
| | generative cell, sperm cells | 2, 3, 11, 12, 13 |
| | male sterility | 3, 11 |
| | anther - pollen dehydration | 4 |
| CLEISTOGAMY | | 4, 9, 11 |
| HETEROSTYLY and/or HETERANTHERY | | 4, 9, 11 |
| FERTILIZATION | male cytoplasmic inheritance | 1, 2, 3 |
| | quiescent zygote | 11 |
| | polyembryony | 1, 2 |
| SEED DEVELOPMENT | embryo-endosperm development | 1, 2, 3, 11 |
| | reserves mobilization | 9, 11 |

(*) Numbers are those of table 1

description and thus has wide application in taxonomy. Palinology is an outstanding example. Recent techniques also permit the observation of more delicate structures with a high water content such as certain stigmas, and structures within the cells. BARNES & BLACKMORE (1984) have developed a method in which the preparation, fixed or fresh, is frozen and fractured, then macerated for periods sometimes exceeding a week, in diluite solutions of osmic acid and then observed by SEM. In this way it is possible to see cell components in three dimensions according to the fracture.

Another recent technique enable the observation by SEM of material embedded for TEM (PACINI *et al.*, 1980). After removing the resin both the internal and external structures of the cell can be

observed. This method is useful because the same block can be seen by SEM, TEM and optical microscopy.

Autoradiography is not a new technique and has not undergone recent modifications, but can still give very interesting results. For example, the different activities of the amoeboid tapetum of *Rhoeo discolor* were detected by SOUVRÉ & ALBERTINI (1982).

Interference contrast is a routine technique often used to check the phase of development of structures such as pollen grains and the female gametophyte in fixed and embedded material (HERR, 1971). Despite its simplicity, this method can give remarkable results and needs not always be used as an auxiliary to other methods.

Fluorescence microscopy has undergone considerable development in the last 10 years because of the availability of many specific fluorochromes (e.g. optical brighteners) and different wavelength filters. The most important techniques for identifying macromolecules are immunocytochemical localization using specific antibodies raised to purified antigens and the use of lectin probes to identify specific sugars in polysaccharides (KNOX *et al.*, 1980; KNOX & SINGH, 1985). In either case the macromolecule is located by a fluorescent marker bound to a lectin or a specific antibody. If an electron opaque antibody is used instead of a fluorescent one, the method may be used in electron microscopy.

Many substances characteristic of plants, such as lignin, chlorophyll, suberin and cutin are autofluorescent and can therefore be observed directly. Autofluorescence studies must be performed on fresh material to avoid washing out the fluorescent compounds during fixing and embedding. Some of the fluorochrome are natural, e.g. alcohol extracts of *Chelidonium majus* stain various cell components. A frequent problem with fluorochrome arises from their incomplete specificity towards certain cell components and the synonymity of the former. For example, there is a type of calcofluor (white M R) which stains cell wall components (HUGHES & MCCULLY, 1975) and one which stains proteins. The former shows up both the pectocellulosic (PAS positive) and callosic (PAS negative) walls.

A fluorochrome called DAPI which stains DNA, has recently come onto the market. It is so sensitive that it can show up even the DNA of prokaryotes, plastids and mitochondria (COLEMAN & GOFF, 1985, HOUGH *et al.*, 1985). A difficulty frequently met in the observation of mature or developing grains of pollen is due to the natural autofluorescence of the sporopollenin which often masks that induc-

ed in the nucleus or cytoplasm. This is avoided by a) embedding the material in fluorochrome permeable resin: in this way the autofluorescence dissipates and is greatly reduced; b) using fluorochromes which fluoresce at different wavelengths from the sporopollein.

«The Study of Plant Structure: Principles and Selected Methods» by O'BRIEN & MCCULLY (1981) can be recommended as an introductory text in the use of fluorescence microscopy. Recent advances in fluorochromes are published in several journals of histochemical techniques. KAPIL & TIWARI (1978a) are the authors of a review of fluorescence microscopy methodology and its application in embryology.

The cryostat is quite an old apparatus in most histochemical laboratories but it is not often used in the study of reproductive structures because they comprise tissues of different consistencies which are difficult to slice. However new supports for embedding materials are making this possible and the cryostat is already being used in pollen research (KNOX *et al.*, 1980). In the last 5 years certain cryoultramicrotomes have made their appearance and begin to give good results although their elaborate techniques do not lend themselves to a great variety of materials.

Microscopy images are always static because the material is dead and stained to bring out its structure. Microcinematography in Biology began to develop in the thirties. Lately a Czechoslovakian worker, Olga Erdelska, has been filming the fertilisation and development of the angiosperm ovule. Her films are not only didactic but an important contribution to the understanding of embryo-suspensor-endosperm relationships (ERDELSKA, 1983).

Embryological studies are usually descriptive rather than quantitative. When the interest is also genetic, sometimes the DNA content of certain nuclei (e.g. of the tapetal cells, pollen grains, suspensor) is measured by cytodensimeter (D'AMATO, 1984). The determination of cell and organelle areas and volumes was extremely tiresome work until quite recently, as it was performed by squared-paper methods or outline-chipping weighting. Nowadays specially programmed microcomputers coupled with camera lucida are widely used. This is not to say that the apparatus does all the work. The optimum use of even the simplest scientific instrument depends on the ability and cultural background of the researcher.

## MAIN TOPICS AND NEW ADVANCES IN EMBRYOLOGICAL RESEARCH

### *The ovule*

Investigations into megaspore polarity have been performed mainly by Rodkiewicz and his coworkers. They have demonstrated, using gametophytes of different origin (monosporic, bisporic, tetrasporic), that the polarization of the megaspore depends on the presence and thickness of the callosic walls separating them. WILLEMSE & BEDNARA (1979) also demonstrated the polarization of the organelles and certain enzymes. It has also been postulated that the nucellus and vascular bundle influence polarity.

The nucleoids are small spheres of RNA released during the two meiotic divisions in both the male and female lines. These structure have been studied in detail in the lily (DICKINSON & WILSON, 1985) and also observed in the olive (PACINI *et al.*, 1985a). It has been proposed that this event serves to free the meiocytes from long term information-carrying molecules thus permitting rapid differentiation of the microspores, and constitutes a point in the life cycle of the organism at which «rationalization» of cytoplasm information can take place (DICKINSON & WILSON, 1985).

The endothelial tapetum bounds the female gametophyte and later on the endosperm, and normally consists of a row of cells. Histologically, this tissue has meristematic, secretory and storage characteristics. Its function is to assists and coordinate ovule growth by channeling the flow of nutrients into precise areas. Excessive proliferation of this tissue in certain hybrids leads to the abortion of the seed. For the above reasons the endothelial tapetum is analogous to that of the anther (KAPIL & TIWARI, 1978b).

The organization of the embryo sac has been studied in detail by Wageningen group (Willemse, van Went, Wilms) in *Petunia, Spinacia, Gasteria* and in some case the morphological changes in the organelles according to stage of development have been described (WILMS, 1980b).

There has been great progress in the last 20 years in knowledge of the development of granule walls. Towards the end of the sixties there was a big debate about the origin of exine. Some said it was of exclusively gametophytic origin and others that it was only sporophytic. At the beginning of the seventies it was generally considered to be of mixed origin, at least in most species. However

the definitive proof of this has not yet been obtained (PACINI *et al.*, 1985b). At the same time both intine and exine with their gametophytic and sporophytic proteins were being studied. These two types of protein of different origin are believed to recognise each other, at least those localized in the pores (PACINI *et al.*, 1981). When there is sporophytic autoincompatibility, these proteins may play a role in pollen-stigma recognition (HESLOP-HARRISON, 1975). Some of these proteins have also been shown to participate in allergic phenomena (HOWLETT *et al.*, 1979). The usually lytic enzymes associated with the pollen wall proteins might help the granule and tube to make their way in the stigma (KNOX, 1984).

With the coming of electronmicroscopy, it was confirmed cytologically that the male gametes of angiosperms are cells which may contain plastids (LOMBARDO & GEROLA, 1968). It was also postulated that if there are plastids, they can be eliminated by degeneration or extrusion during the maturation of the granule or the growth of the pollen tube (CLAUHS & GRUN, 1977; MOGENSEN & RUSCHE, 1985). These cells also present bundles of microtubules which form a strong skeleton (CRESTI *et al.*, 1984). In mature pollen the generative cell or spermatic cells and the vegetative nucleus may be arranged in different ways i.e. united with or distant from generative cell extensions which embrace the vegetative nucleus or from lobes of the negative nucleus which embrace the spermatic cells (RUSSEL, 1984; MCCONCHIE JOBSON *et al.*, 1985).

Male sterility which has important practical applications has been the subject of much study (DRISCOLL, 1986). Various types of male sterility are known and substances (gametocides) have been identified which have a negative effect on pollen development (COLHOUN & STEER, 1985).

The problem of dehydration and dehiscence of the anter was tackled at the beginning of the century and was subsequently set aside. Two hypotheses were formulated, namely the evaporation of the contents of the locule through cracks in the cuticle (MEPHAM & LANE, 1969) or reabsorption (LINSKENS, 1973).

Changes in shape and volume of the pollen grain due to dehydration (in the anter) and hydration (on the stigma) were studied in general terms (PACINI & FRANCHI, 1984) and a technique for studying the hydration of the granule in vitro proposed (PACINI, 1986).

Cleistogamy studies have received much attention recently, mainly by Elizabeth Lord who has performes systematic and histological

investigation of the phenomenon (LORD, 1981). Subsequently she examined the physiological aspects of the question and determined the triggering factors. The environmental causes of temporary cleistogamy have also been investigated and mathematical models devised which take into account habitat and reproductive strategy (SCHOEN & LLOYD, 1984). PHILBRICK (1984) and ANDERSON (1980) described two very complex cases of cleistogamy in which the pollen tubes penetrate the filament and reach the ovules via the receptacle unlike all the other cleistogamic species in which the tubes penetrate the stigma-style.

Heterostily is a phenomenon that was studied in depth by DARWIN (1888). With the advent of the electron microscope (SEM and TEM) and histochemistry, many advances have been made in the understanding of the morphology and the histological and physiological differences between stigmas (SCHOU & MATTSSON, 1985) and pollen grains (STEVENS & MURRAY, 1982; SHIVANNA *et al.*, 1983). The progamic phase and phenomena of autocompatibility have received much attention, especially the histological and morphological aspects and recenlty there has also been a molecular approach (CLARKE *et al.*, 1985).

The TEM has also helped our understanding of fertilization which has been described in detail in *Gossypium, Plumbago zeylanica* (RUSSEL, 1982), *Petunia, Capsella, Helianthus, Linum, Spinacia* (WILMS, 1981). The passage of the spermatic nuclei from the synergid to the egg cell or proendospermatic cell has been observed in even fewer species and thus means and pathway are still unclear. A popular hypothesis is that the two male gametes are histologically very different (i.e. in organelle content). In species such as *Plumbago zeylanica,* quantitative and qualitative differences in the organelles have been observed in sperm cells; this make it appears likely that even the female gametes to be fertilized are predetermined (RUSSEL, 1985).

A group of Australians (KAUL, ROUSE, KNOX & WILLIAMS, 1985) has studied the events preceding and following fertilization in the genus *Rhododendron.* While the endosperm is dividing the young zygote shrinks in volume and becomes surrounded by a callosic wall which begins its formation in the area adjacent the degenerating syngergid. This finding is of great importance because it demonstrates a type of isolation similar to that which occurs in microspores after meiosis.

Even the development of the embryo and the endosperm have been widely investigated, especially in plants of agricultural interest. Some of the studies which examine the relationships between the embryo and the surrounding tissues include that of SCHEL and Coworkers (1984) and SMART & O'BRIEN (1983). The mode of cellularization of the nuclear endosperm has been the subject of attention especially in agricultural species such as wheat, but the results are sometimes contradictory because the variety of *Triticum aestivum* studied has not always been the same (FINERAN *et al.*, 1982).

In conclusion, embryological research has made many advances in recent years especially in pollen biology. This is certainly because the study of the male gametophyte is much easier than the female, which requires the use of serial sections. Electron microscopy has provided a considerable amount of information and has often been combined with histochemical or audioradiographic techniques. When an argument has been approached with a variety of techniques, better results have been achieved in most cases.

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