Embryology of Angiosperms

Edited by B. M. Johri

With 278 Figures

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Preface

Since the publication of *Recent Advances in the Embryology of Angiosperms* (ed. P. Maheshwari) in 1963, there has been phenomenal progress in almost all areas of reproduction in flowering plants. This progress has resulted from investigations based on optical, electron (TEM, SEM), fluorescence, phase contrast, and interference (Nomarski effect) microscopy, cytochemistry, histochemistry, physiology and biochemistry. However, all this knowledge is scattered in a large number of periodicals and it has become a formidable task for both teachers and students to collect the necessary information. Hence this volume *Embryology of Angiosperms* – an advanced treatise comprising 16 chapters written by specialists who have devoted years of study to the subject.

The significant advances of the last 20–30 years have been discussed. The authors have raised several questions to seek new information, and have made appropriate mention of many unsolved problems. The text is adequately illustrated with line drawings and half-tones, including electron micrographs.

Every effort has been made to present a comprehensive up-to-date account. I have earnestly endeavoured to achieve uniformity in the format. Suggestions for further improvement would be most welcome.

I have no doubt that post-graduate and research students will find *Embryology of Angiosperms* a good source material. To those teaching "reproduction in flowering plants", the volume offers a fount of ready-made material.

July 1984 B. M. JOHRI

Acknowledgements

Through Dr. G.A. Nogler, Dr. Konrad F. Springer invited me to prepare an English translation of Professor A. Rutishauser's book *Embryologie und Fortpflanzungsbiologie der Angiospermen*. My experience of teaching undergraduate and postgraduate students for over three decades at the Universities of Agra, Rajasthan and Delhi has provided a deep insight into the various aspects of plant embryology. Also, over a span of almost 50 years, I have had unique opportunities to discuss problems of angiosperm embryology with eminent embryologists during my numerous visits both in India and abroad, and at international meetings. The idea of editing a volume consisting of contributions on recent developments in the embryology of angiosperms emerged out of these interactions. Dr. Springer readily agreed to my suggestion, and I am deeply indebted to him.

Professor J. Heslop-Harrison has done me a great favour in agreeing to prepare the "Foreword" to this volume.

I am grateful to the authors who accepted my invitation to write the chapters. The manuscripts were read by my colleagues in the University of Delhi and I especially thank Professor N.S. Rangaswamy, Dr. N.N. Bhandari, Dr. M.R. Vijayaraghavan, Dr. K.R. Shivanna and Dr. S. Natesh for offering valuable suggestions. I greatly appreciate the help rendered by Dr. K. B. Ambegaokar, who redrew several diagrams, reassembled many plates of illustrations, and prepared the indices.

Professor Dr. G. Melchers (Tübingen) provided photographs of the tomato + potato somatic hybrid. Professor Dr. E. Battaglia (Rome) sent a reprint containing the original diagrams of Fritillaria type of embryo sac. Professor Dr. F. Pospisil (Prague) sent photographs of pollen embryo sacs. Professor Dr. O. Erdelská (Bratislava) prepared an illustrated write-up on the role of microcinematography in embryology. Dr. Scott D. Russell (Norman) sent me his unpublished manuscripts, and electron micrographs of male gametes and fertilization in *Plumbago*. Dr. R. Wunderlich (Vienna), Dr. M. Luxova (Bratislava), Dr. L. Ahlstrand (Göteborg) and Dr. B.A. Fineran (Christchurch) made available the literature needed. I am much obliged to all of them for their gracious help.

The facilities at the Department of Botany, University of Delhi, made it possible to prepare the *Embryology of Angiosperms*, and I am grateful to Professor S.C. Maheshwari, Professor H.Y. Mohan Ram, Professor R.N. Kapil and Professor R.N. Chopra (present Head of the Department) who have been continuously looking after my interest since I

retired in 1974. I have received much inducement to complete the book expeditiously from my wife Raj, my daughter-in-law Meera, and my son Lalit.

My sincere appreciation is extended to Mr. R. K. Gupta for typing several chapters, and to Mr. Krishan Lal for preparing some illustrations.

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July 1984 B. M. JOHRI

Foreword

Thirty-four years have elapsed since the publication of the late Professor P. Maheshwari's text, *An Introduction to the Embryology of Angiosperms*, a work which for many years served as an invaluable guide for students and a rich source book for research workers. Various texts dealing with sections of the broad spectrum of topics encompassed by Maheshwari in his book have appeared in the interim, but a compendious modern work dealing with the whole field has been lacking. This present volume splendidly meets the need, and it is altogether fitting that Professor B. M. Johri, long an associate and close colleague of Professor Maheshwari and himself a prolific contributor to the subject, should have undertaken the task of editing it.

When Maheshwari wrote, it was still feasible for one author to handle the subject, but today even someone with his fine breadth of vision and depth of understanding could not, alone, do it justice. So the effort has to be a collaborative one; and Professor Johri's achievement has been to bring together a team of authoritative collaborators, assign them their responsibilities, and put them to work to produce a text as integrated in its treatment as the diversity of the subject would allow. The product vividly illustrates the advances that have been made in the study of angiosperm reproductive systems in the last 30 years, and the book is surely destined to become the new standard for student and researcher alike.

Like many surveys of its kind, the text inevitably shows just how dependent progress during the last few decades has been on the advent of new technologies. The great observers of earlier generations reached the limits set by the resolution of the optical microscope in their investigations of the cytological and karyological phenomena associated with plant reproduction, and were restricted further by the preparation techniques they had available. Their modern successors have been blessed with new extensions of vision – on the one hand through the advent of electron microscopy, and on the other, through the further technical development of optical microscopy, marked, for example, by the introduction of phase and differential interference contrast, fluorescence techniques and image-processing systems. At the same time, specimen handling methods have improved apace, and the crumbling of hitherto stoutly maintained interdisciplinary barriers has meant that many workers today have gained a new competence to interpret what they observe through the support of physiological and biochemical experimentation. All this adequately accounts for the flood of new information. Yet, if one

message emerges from the present text as a whole – urged by author after author – it is that we are now but on the threshold of a new wave of discovery. No living group shows the diversity of reproductive method to be seen in the angiosperms; and no task in the whole field of biological research is more important for mankind than working the systems out and finding how to manipulate them for practical ends, for after all the flowering plants provide the indispensable basis of human life on earth. This volume is not only an epitome of knowledge, but an invitation and a challenge – a challenge, particularly, for the next generation of workers, some of whom may well find in its pages good reason for ignoring the facile appeal of test-tube biology in favour of making their contribution towards finding out more about how real plants go about the job of reproducing.

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1 Embryology: Then and Now

B. M. JOHRI and K. B. AMBEGAOKAR

1.1 Introduction

Maheshwari (1950) has summarized the history of development of our knowledge of embryology of angiosperms. The highlights are presented here. Sexuality in angiosperms has been known since the third century B.C. when the Arabs and Assyrians used to perform the ritual of artificial pollination of the date palm. They were not, however, aware of sexuality as such, nor what followed after dusting the female flowers with male flowers.

Camerarius (1694), at the University of Tübingen, established sexuality in *Mercurialis annua*. Kölreuter (1761), also at Tübingen, demonstrated that seeds could be produced by artificial pollination in *Dianthus, Hyoscyamus, Matthiola,* and *Nicotiana*. However, he hardly visualized the great importance of the technique of artificial pollination in plant breeding and crop improvement.

A little later, Amici (1824), an Italian astronomer and also a microscope maker, while studying the stigma of *Portulaca oleracea,* observed that a pollen grain burst forth into a tube. He (Amici 1830) made a detailed examination of *Hibiscus syriacus* and *P. oleracea,* and pointed out that the pollen tubes had grown, bit by bit, and came in contact with the ovule, one for each ovule.

In a meeting held at Padua in 1842, Amici emphasized that the embryo did not originate from the tip of the tube, but from a portion of the ovule which had already existed before the entry of the pollen tube. Finally, in *Orchis,* Amici (1847) reported that in an embryo sac a "germinal vesicle" (egg cell) was present before the entry of the pollen tube, and the embryo originated not from the tip of the tube, but from the germinal vesicle.

Schleiden (1837, 1845) confirmed Amici's work about the growth and entry of the pollen tube into the ovule through the micropyle. However, he still maintained that the tip of the pollen tube becomes the "embryonal vesicle", and undergoes a number of divisions to form the embryo.

Hofmeister (1849) confirmed Amici's observations, and pointed out that in all the 38 species belonging to 19 genera of angiosperms examined by him the embryo originated from a pre-existing cell in the embryo sac, and not from the tip of the pollen tube. His observations and description were convincing, and were confirmed by other workers. Thus ended the fierce controversy between Schleiden and his opponents.

Hofmeister (1849) also studied the formation of tetrads during microsporogenesis in *Tradescantia;* development and organization of female gametophyte, and formation of Cellular type of endosperm in *Monotropa hypopitys.*

Strasburger (1879) worked out the development of female gametophyte in *Polygonum divaricatum,* and observed the formation of linear tetrad, establish-

ment of polarity at binucleate stage, and organization of mature eight-nucleate embryo sac. In 1884 he reported the widespread occurrence of binucleate (bicelled) pollen grains in several taxa, the formation of vegetative and generative cell in a pollen grain, and the formation of male gametes. But a significant research was the discovery of syngamy in *Monotropa*. In *M. hypopitys* he (Strasburger 1884) observed the tip of the pollen tube with a vegetative nucleus and two male gametes, the entry of the pollen tube through the micropyle, and the process of fusion of one male gamete with the egg nucleus. He also mentioned that the cytoplasm of the pollen tube does not enter into the egg cell.

Strasburger (1878) traced the development of adventive embryos from the nucellus in *Citrus aurantium, Coelebogyne* (*Alchornea*) *ilicifolia, Funkia* (=*Hosta*) *ovata*, and *Nothoscordum fragrans*.

Nawaschin (1898) discovered double fertilization in *Fritillaria tenella* and *Lilium martagon*. He observed the union of one male gamete with the egg cell (syngamy) and the fusion of the other with the two polar nuclei (triple fusion). Guignard (1899) also observed double fertilization in *Lilium* and *Fritillaria*, and illustrated his findings with magnificent illustrations.

Hanstein (1870) investigated the development of embryo of *Capsella bursa-pastoris*. He could follow the formation of quadrants, octants, delimitations of three germinal layers, and even hypophysis. In monocotyledons he studied the development of embryo of *Alisma plantago*.

With all the basic facts about reproduction in flowering plants known by the end of the nineteenth century, embryology of angiosperms became an established discipline. To begin with, most investigators looked for specific stages of development. With stage-oriented approach, some of the more interesting features were missed, and this often led to misinterpretations.

By the 1920's a great deal of embryological information had accumulated, and it prompted Schnarf (1931) to publish the first comparative account under the title „Vergleichende Embryologie der Angiospermen".

With improvements in microscopy, and refinements in techniques, there have been significant advances in our knowledge of embryology. These are dealt with in different chapters.

Chapter 1 is devoted to techniques to study embryology, nutrition of ovule and seed, and some unusual and interesting features in the embryology of angiosperms.

1.2 Techniques to Study Embryology

The earlier investigations were carried out by selecting such taxa as had a more or less transparent ovary wall and ovules. The larger ovules were dissected to follow the later stages of development of endosperm and embryo. All stages of microsporogenesis and male gametophyte could be studied from squash preparations. The "rocking" microtome made it possible to cut sections, but serial sections were rather difficult; the "rotary" microtome made it much easier to cut the sections swiftly and serially. Paraffin wax is still used as an embedding medium,

though plastic-embedded materials yield very thin sections. Ultratome is used for cutting even thinner sections (materials embedded in resins) for electron microscopy.

With advances in microscopy, and use of cyto- and histochemical techniques, it became possible to study major features of wall structure, nuclear number and behaviour during megasporogenesis and in the female gametophyte, development of endosperm, embryo, and seed, and distribution of metabolites at various stages of development. The electron microscope opened up an entirely new field of observation and interpretation of fine structure. Examples of such studies are detailed in several chapters.

Polarizing optics can be used for studying starch grains, cell walls and crystalline inclusions in cells. The distribution of crystals and starch grains in fresh and cleared material is easily determined.

Phase contrast microscopy provides an extremely sensitive technique for the examination of living cells, and weakly stained (IKI) specimens show a remarkable improvement. The spectacular results achieved by Molè-Bajer and Bajer (1968) on mitosis in living cells of endosperm owed their quality to the very thin layers of flattened cells.

Fluorescence microscopy has demonstrated the presence of callose during microsporogenesis (see Fig. 2.18A, B), and interaction between pollen and stigma in compatible and incompatible reactions. Sporopollenin in the exine of pollen grains is intensively autofluorescent. In the absence of autofluorescence, fluorochromes are used to excite fluorescence (see O'Brien and McCully 1981).

Nomarski optics (differential interference contrast microscope) are used for thick specimens, and the image shows excellent relief (see Fig. 10.6). Herr (1982) has used such optics to study megasporogenesis in cleared wholemounts of ovules of *Planera aquatica* and *Cassia occidentalis*.

The scanning electron microscope has been used to great advantage in the study of surface structures (see Figs. 12.11 and 12.12). In *Vaccinium* (Brisson and Peterson 1975) the freeze-fractured endosperm haustorium (Fig. 7.12A–C) revealed conspicuous wall ingrowths (characteristic of transfer cells) which provide a much larger surface for absorption of nutrients.

The transmission electron microscope has significantly advanced our knowledge of a wide range of structures. The wall ingrowths in synergids (Fig. 4.8), antipodal cells (Fig. 4.16 A, B), and suspensor (Fig. 8.24) are only a few examples.

Bajer (1955, 1957) worked with living endosperm for studying mitosis. The endosperm cells lack cellulose walls and, hence, the penetration of various chemicals is facilitated. The phases of mitosis are comparatively better seen in the endosperm cells. Some of the monocotyledonous taxa (*Clivia, Colchicum, Haemanthus, Iris*) have thin, partially transparent ovules and are suitable for squash studies.

After 3–6 weeks of pollination endosperm cells show suitable stages of mitotic division. On a large coverslip (1–2 mm thick), within a ring of vaseline of 2-cm diameter, hot agar with sugar solution (agar 0.4%–0.5%, glucose 3%–4.5%) is smeared. The endosperm (with liquid contents) is pressed out of the ovule on to the agar, and covered with another coverslip. The edges of the coverslips are sealed with vaseline or liquid paraffin. By carefully pressing the coverslip the liv-

ing cells and mitotic spindles are flattened to chromosome thickness. This preparation is inverted and mounted on a suitable metal holder.

Bajer analyzed mitosis from the commencement to the end of the process. The preparations were examined under phase contrast microscope, and photographed.

Molè-Bajer (1955) studied the effect of chemicals, which have an inhibitory effect on respiration, on living endosperm. The treatment was given directly to the excised endosperm, or through agar-glucose medium, or by injection into the embryo sac before excising the endosperm.

Herr (1971) has suggested a clearing squash technique for the study of sequential development of anthers and ovules. The pistils are fixed for 24 h in formalin-propionic-50%-ethanol, or Randolph's modified Navashin fluid (see Johansen 1940), and stored in 70-% ethanol. The material is then treated with a clearing fluid (for 24 h) which contains 85%-lactic acid: chloral hydrate: phenol: clove oil: xylene (2:2:2:2:1 – by weight). (For the sake of convenience we designate this as "Herr's fluid" although the author designated it 4½-clearing fluid.) From these transparent pistils the ovules are dissected, transferred to absolute ethanol, and upgraded in mixtures of ethanol: xylene (3:1, 2:2, 1:2). Finally, the ovules are mounted in "Piccolyte", or "Permount", on specially prepared micro-well slides (designated Raj-slides). This is prepared by fixing, with permount or balsam, two thick cover-slips, 1 cm apart, on a microscopic slide. Originally, Muir et al. (1958) had improvised such a micro-well slide for growing single cells. It is placed on a warming plate at 50 °C for 3 days to harden the mounting medium of the two cover-slips. The ovules (with the clearing fluid) are placed in between the cover-slips, and covered by another cover-glass which rests on the two affixed cover-slips. The clearing fluid gives suffcient transparency to study the cellular structure of the ovules, under phase contrast optics.

Sometimes, a pre-treatment with lactic acid (85%) for 24 h is essential. Smith (1973) placed the ovules of *Cornus* in lactic acid for 4 days before treating them with the clearing fluid. Otherwise, treatment with 10%-potassium hydroxide solution for 2 min, followed by dehydration with 95%-ethanol, is necessary.

Herr (1973) further suggested the use of three modified mixtures for the study of ovules with interference (Nomarski) optics.

1. Iodine-potassium iodide (IKI) + Herr's fluid – prepared by adding 100 mg iodine, 500 mg potassium iodide, and 9.0 gm of Herr's fluid. The ovules pretreated with Herr's fluid are selected under phase contrast optics, and transferred to IKI + Herr's fluid. This mixture increases the contrast of cell structure, sharpens the features, and detects the presence of starch.
2. Benzyl-benzoate (BB) + Herr's fluid – composed of lactic acid: chloral hydrate: phenol: clove oil: xylene: benzyl benzoate (2:2:2:2:1:1 – by weight). The ovules are treated with this mixture for about 2 weeks. While the cytological features, such as nuclei, nucleoli, and vacuoles do not stand out, the cell outlines of the ovules become sharply defined.
3. In contrast to the previous two mixtures, potassium permanganate + Herr's fluid is unstable, and has to be prepared for immediate use – 3 mg potassium permanganate are dissolved in 1 gm Herr's fluid. The treatment with this fluid

sharpens the structure of ovule, particularly the cell layer topography under phase contrast or interference (Nomarski) optics.

Herr's (1982) procedure to make permanent preparations of ovules treated with BB + Herr's fluid is also worthy of attention. A satisfactory mountant is the epoxy resin combination which is used in electron microscopy (Spurr 1969). Ten drops of vinylcyclohexene dioxide are combined with 6 drops of diglycidyl ether of polypropylenegiycol, and 26 drops of nonenyl succinic anhydride. The mixture is freshly prepared and a graded series with clearing fluid: epoxy medium – 3:1, 2:2, 1:3 – is used in upgrading the treated ovules. The ovules cleared for 24 h are passed through this graded series with 15 min exposure to each mixture and, finally, mounted in epoxy medium. A drop of cure accelerator (dimethylamino-ethanol) is added to the medium, and the ovules are mounted on micro-well slides. The preparations are kept in an oven at 60 °C for 24 h, and then observed with phase contrast or interference (Nomarski) optics. The Spurr epoxy mountant avoids the shrinkage artifact.

1.3 Embryo Sacs in Anthers

1.3.1 *Leptomeria*

While the sporogenous cells in anthers normally develop into microspore mother cells, undergo meiosis, and give rise to tetrads, microspores, and pollen grains, in *Leptomeria billardierii* Ram (1959a) observed a rather unusual feature. Some of the sporogenous cells enlarge, and develop into an uni- or binucleate embryo sac-like structure (Fig. 1.1 A). Two subsequent mitotic divisions result in the formation of an eight-nucleate "embryo sac". These nuclei even organize like a typical embryo sac with three-celled egg apparatus, two polar nuclei, and three antipodal cells (Fig. 1.1 B). This phenomenon has not been reported in any other angiosperm so far.

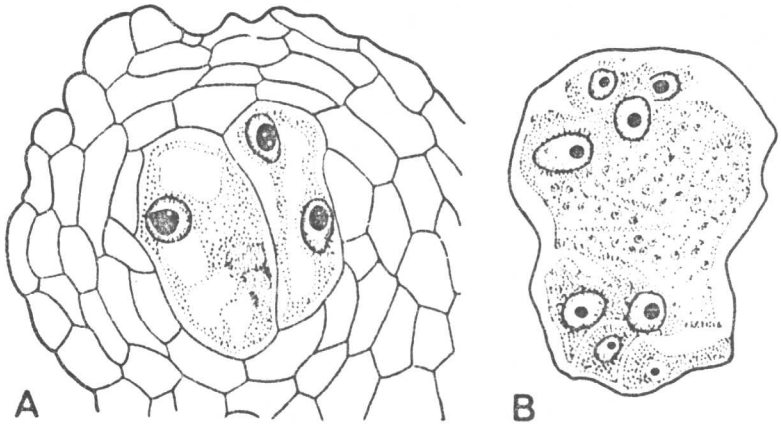

Fig. 1.1 A, B. *Leptomeria billardierii*. **A** Transection of anther lobe; of the two sporogenous cells, one is a binucleate "embryo sac". **B** Organized eight-nucleate "embryo sac". (After Ram 1959a)