Fertilization in flowering plants – What is new?

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The process of fertilization is more complex in flowering plants than in other groups of plants. Following successful pollination, a pollen grain germinates on the stigma and the resulting pollen tube grows through the stigma and style and carries the two male gametes into the ovule. Eventually the pollen tube enters the embryo sac and releases the male gametes. One of the male gametes fuses with the egg to form the zygote and the other with the central cell to produce the endosperm, thus completing the process of double fertilization, a unique feature of flowering plants. The zygote gives rise to the embryo; the central cell-derived endosperm nourishes the embryo. Thus, fertilization in flowering plants takes place deep inside the ovule and imposes technical problems for basic studies as well as for effective experimentation. Because of this constraint, our knowledge of fertilization in flowering plants has been rather meagre and lags far behind that in animals and some lower plants in which fertilization is external and can be observed under the microscope. One of the main objectives of the experimental embryologist has been to achieve fertilization *in vitro* by using isolated egg and sperm cells, and development of the *in vitro* formed zygote into embryo. This would enable effective experimentation on fertilization in flowering plants comparable to that in animals and lower plants.

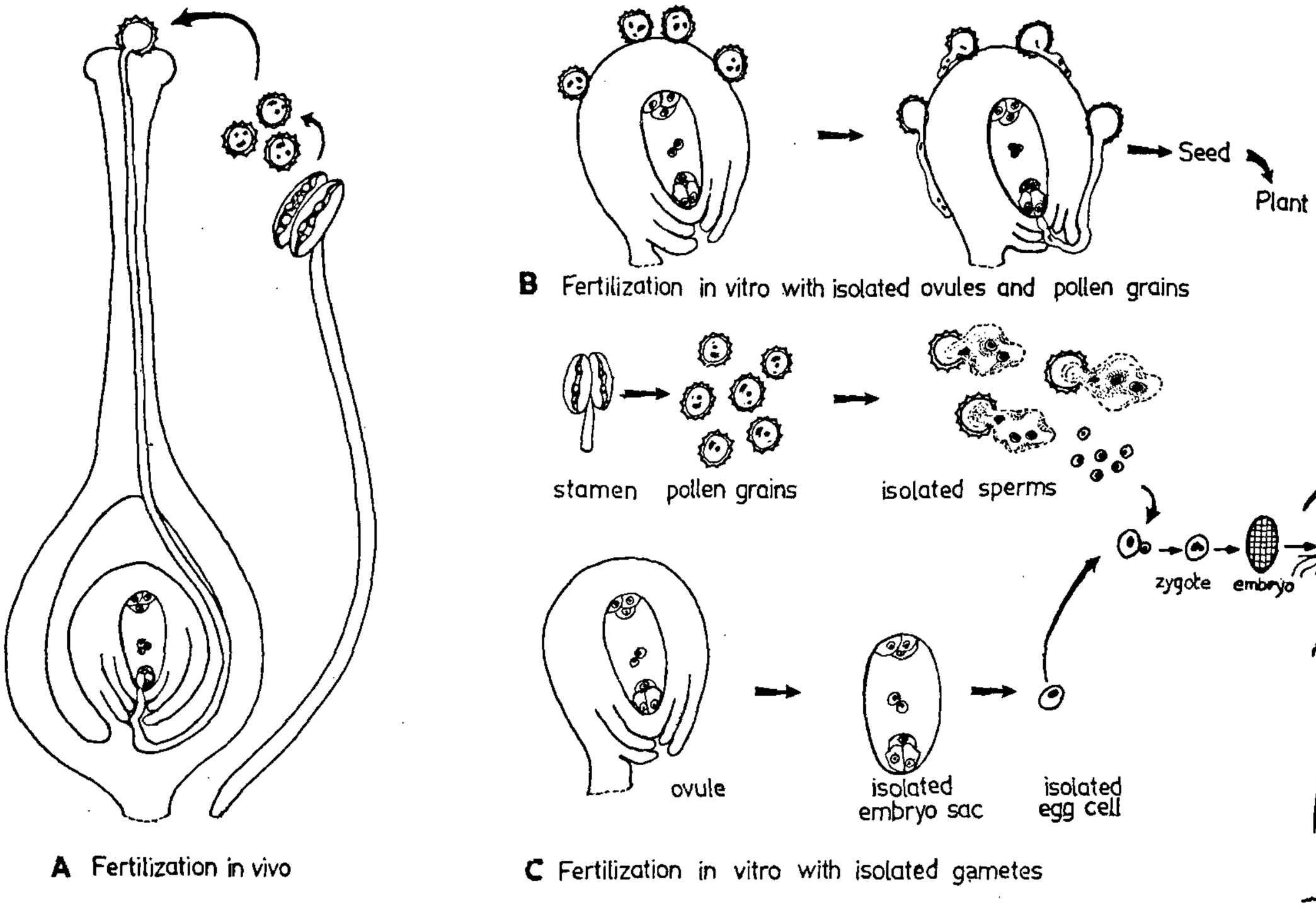
A beginning on these lines was made over 30 years ago in the laboratory of the late Panchanan Maheshwari at the University of Delhi. Maheshwari and his associates[1] cultured isolated groups of ovules and pollen grains of opium poppy (*Papaver somniferum*) together on a nutrient medium. Pollen germination, pollen tube entry into ovules and double fertilization proceeded normally; the fertilized ovules developed into viable seeds; this research report in *Nature* was covered in leading newspapers in India and abroad. Subsequently the technique was refined to minimize injury to the ovules and to overcome self-incompatibility[2]. Although the technique has been successfully used over the years to overcome crossability barriers in several species[3,4], progress in advancing the technique to achieve *in vitro* fertilization with isolated egg and sperm cells was hampered for want of techniques to isolate viable male gametes (sperms) and eggs and fuse them *in vitro* to achieve fertilization.

Rapid advances in protoplast technology and somatic hybridization during the 1970s and the 1980s gave new impetus to studies on *in vitro* fertilization. Recent progress in recombinant DNA technology, which made the egg, sperm and zygote very attractive systems for genetic transformation, compounded the interest in research on *in vitro* fertilization. Studies were initiated the world over on isolation of sperms and embryo sacs in the early 1980s and the progress was overwhelming[5]. Basically two procedures were employed to isolate sperms from pollen grains: mechanical method and osmotic shock method. In the first, the pollen grains are ruptured mechanically by grinding them in isolation medium, and in the second by incubating the pollen grains in a hypotonic solution. The debris is removed through filtration and the sperms are then separated through density gradient centrifugation. Embryo sacs are generally isolated by combining enzymic maceration of ovules with microdissection. Subsequently the technique was further refined to isolate protoplasts from constituent cells of the embryo sac (egg, synergids and central cell). Thus, by 1990, protocols were available for isolation of viable protoplasts from sperms and eggs of many plant species, and the stage was set to make serious attempts to achieve *in vitro* fertilization.

The pioneering success of fusing isolated egg and sperm cells *in vitro* was accomplished in maize by Kranz and his associates at the University of Hamburg in 1991 (ref. 6). It took another 2 years to standardize the conditions to realize embryogenesis and plantlet formation from *in vitro*-formed zygotes[7]. The fusion of gametes was performed under the microscope in microdroplets (2 µl) of fusion medium covered with mineral oil. Isolated egg and sperm cells held in microcapillaries were transferred to the fusion droplets by using a computer-controlled dispenser. The egg and sperm cells were aligned electrophoretically or mechanically with microneedles and fusion was induced by giving one or a few short pulses of direct current. The fusion was completed in <1 s and karyogamy occurred in <1 h from fusion. As in somatic protoplast fusion, in gametic fusion also the composition and osmolarity of the medium were critical. The *in vitro*-formed zygotes were cultured on a semipermeable membrane placed on fast growing nonmorphogenetic cell suspension cultures derived from maize embryo or microspores. In these nurse cultures the zygotes established polarity, showed as high as 85% division, and gave rise to globular structures, proembryos and transition phase embryos comparable to seed embryos *in vivo*, and eventually to fertile plants.

Next, *in vitro* fusion of isolated gametes in maize was achieved not with electric pulse but in the presence of high calcium[8] and high alkalinity (pH 11) (ref. 9); also, attempts were made to microinject sperm nuclei into isolated embryo sacs[10]. Furthermore, it has been possible to regenerate whole plants of maize by culturing the *in vivo*-formed zygote[11]. Recently sperm cell has been fused with the central cell also; the resultant fusion product was capable of growth comparable to endosperm[12]. A further milestone has been the demonstration that the fusion product of maize egg and sperm of the related genera, wheat and sorghum, underwent divisions, but not that of maize egg and sperm of the unrelated genus *Brassica*. Even more significant has been that the fusion product of two maize eggs failed to undergo divisions[11]. Most of the achievements of the past decade on *in vitro* fertilization have been with maize, and there is no compelling reason to disclaim that the various techniques employed will not work equally well with other higher plant species.

The advances on *in vitro* fertilization and embryogenesis have opened up a vista of experimental studies. Some of the fundamental problems that can be effectively researched on (using the technique of *in vitro* fertilization) are: (a) mechanisms of recognition, adhesion



A Fertilization in vivo

B Fertilization in vitro with isolated ovules and pollen grains

C Fertilization in vitro with isolated gametes

Figure 1. Diagrammatic representation of fertilization *in vivo* and *in vitro*.

and fusion of gametes, (b) parthenogenesis, (c) polyspermy, (d) establishment of polarity in the zygote, and (e) genetic regulation of early embryogenesis.

Indeed, *in vitro* fertilization and embryogenesis provide a very powerful biotechnological tool. Besides its potential application in overcoming incompatibility barriers, its application in genetic transformation studies using the sperm, or the egg, or the zygote is very promising. Studies have already been initiated on these lines; of late a paper from Duma's laboratory (Lyon, France[13]) describes optimal conditions for efficient microinjection of DNA into cultured maize zygotes and transient expression of the microinjected genes in the zygote. It is needless to emphasize that researches on fertilization in flowering plants have entered an exciting phase; in the coming years rapid progress not only in understanding the process but also in exploiting it for crop improvement will accrue, and indeed many a young plant scientist will find the work a much rewarding avocation. And who knows, *in vitro* fertilization may lead to raising a hybrid between the two groups of seed plants in not too distant a future!

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