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TEST-TUBE FERTILIZATION OF OVULES

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Ladies and Gentlemen,

I must express my grateful appreciation to the National Institute of Sciences for the award of the Hora Memorial Medal for 1963. I consider it to be not so much a recognition of my own work but that of my pupils and the general progress that plant embryology has made in India. In this my thoughts go back to Dr. W. Dudgeon of Allahabad and Dr. M. A. Sampathkumaran of Bangalore who laid the foundations of this branch of botany in our country and furthered its cause in every possible way during their lifetime. To both of them, particularly Dr. Dudgeon, I owe much for the inspiration I received in my own work.

It is well known that the flower comprises four cycles of organs: sepals, petals, stamens and carpels. Of these the sepals are mainly protective; the petals are often brightly coloured and serve to attract insects which incidentally bring about pollination; the stamens produce the pollen; and the carpels bear the ovules inside them. The stamens and the carpels are known as the essential organs of the flower. This does not, of course, mean that the sepals and petals are unessential but that they are comparatively less important.

For a long time the function of the pollen remained unknown and there was not even a recognition of sex in plants. The reason for this is not difficult to understand. In animals the male seeks the female and the difference in the two sexes was obvious to our ancestors. With regard to most of our economic plants the condition is different because here the flower contains both stamens and carpels. It was, therefore, conjectured that sex is absent in plants and the fruit and seed are generated out of superfluity of nutrition.

The first rays of light on this point probably appeared in Egypt and the countries comprising Central Asia, such as Iraq, Iran and Babylon. Here the most important economic plant of those days was the date. In this the trees are of two separate sexes. Some are male and produce the pollen; others are female and produce the fruit. It was quickly realized by the ancient Egyptians and Babylonians that approximately half the trees remained sterile and only the other half produced fruit. However, fruit formation appeared to be

aided in some manner by the 'sterile' trees which produced only the yellow dust or pollen. The Egyptians were clever enough to realize that they could economize by destroying most of the male trees and preserving the pollen from a few to be dusted over the female flowers. For this they used to have a special ceremony every year in which the high priest carried a basket full of the male inflorescences in the left hand and dusted the pollen over the female flowers with his right hand. Experience showed that this ensured a good set of fruit. In spite of the accounts written by the great traveller, Herodotus, in the fifth century B.C. the Greek and Roman philosophers failed to draw any clear concept of sex in flowers. Even in the middle ages many persons considered the pollen to be a waste product or the excreta of flowers, and it was only in the year 1694 that Camerarius performed some critical experiments and demonstrated that fruit and seed formation could not take place unless the pollen has previously prepared the ovary. He concluded: "In the plant kingdom, the production of seed, which is the most perfect gift of nature and the general means of maintenance of the species, does not take place unless the anthers have previously prepared the young plant contained in the ovary." Subsequent experimenters confirmed this although none of them was clear about the precise manner in which the pollen stimulated the development of the fruit and the seed.

The first microscopic observations of real significance on this subject were made by an Italian mathematician, astronomer and microscope-maker, named Giovanni Battista Amici (1824), who discovered the pollen tube and later (1830) traced it to the mouth of the ovule. Schleiden (1837) confirmed this in many other plants and established the occurrence of the pollen tube in the styles and ovaries of angiosperms as a general feature. However, his unbounded enthusiasm for discovery and an undue keenness to be the first in everything led him into a peculiar misconception, for he stated that the embryo originated directly from the tip of the pollen tube. He and his supporters drew hundreds of figures in support of this view. Amici contended that the embryo arose from a cell already existing inside the embryo sac although the pollen tube admittedly stimulated its development. To this Schleiden angrily retorted: "Solche Präparate sind ohne Zweifel aus dem Kopf gezeichnet."

The heated controversy which was generated between Amici and Schleiden and their many supporters on either side was settled only in the middle of the nineteenth century when a brilliant German botanist, named Wilhelm Hofmeister (1849), showed very clearly that the embryo sac contains several nuclei and that one of these is stimulated to develop into the embryo by a liquid substance discharged from the pollen tube. Earlier, the Imperial Institute of the Netherlands at Amsterdam had offered a handsome prize to anyone who could settle this controversy and clarify the position but unaware

of Hofmeister's work the judges awarded it to one, Dr. H. Schacht, who worked under the same misconceptions which occupied the mind of Schleiden.

When Amici died in 1863, von Mohl wrote an appreciation of his work in which he very appropriately commented as follows: . . . "Es ist jetzt, nachdem wir wissen, dass die Schleiden'sche Lehre ein Irrlicht war, lehrreich, wenn auch betrübend zu sehen, mit welcher Leichtgläubigkeit das Unrichtige für wahr gehalten wurde, wie die einen, auf eigene Untersuchungen vollkommen verzichtend, mit theoretischen Gründen das Phantom herausputzten, die anderen, welche das Mikroskop zur Hand nahmen, durch ihre vorgefasste Meinung geblendet zu sehen glaubten, was sie gar nicht sehen konnten und durch Hunderte von Zeichnungen, welchen nichts als die Wahrheit fehlte, die Richtigkeit der Schleiden'schen Lehre als über jeden Zweifel erhaben darzustellen suchten, und wie eine Akademie durch Krönung einer solchen Arbeit einen neuen Beweis für die alte, namentlich in unserer Wissenschaft seit einigen Decennien wiederholt so glänzend gemachte Erfahrung lieferte, wie wenig Preisaufgaben geeignet sind, die Lösung einer zweifelhaften wissenschaftlichen Frage herbeizuführen."

In spite of his very thorough work Hofmeister had no clear knowledge of the contents of the pollen grain and pollen tube and it took several years before these were correctly identified. This is not surprising because there were no microtomes those days to cut thin and serial sections nor any of the dyes which we now use to stain them. It was only in 1884 that Strasburger was able to show the existence of the vegetative nucleus and the two male gametes in the pollen tubes of *Monotropa*. He also demonstrated that one of the male nuclei fused with the nucleus of the egg and it was this which constituted the essence of fertilization. Nawaschin (1898) and Guignard (1899) completed the story by their discovery of 'double fertilization'. Henceforth it became clear that in the angiosperms there are two fusions: (1) one between the egg and the sperm, resulting in the zygote and then the embryo; and (2) the second between the remaining sperm and the two polar nuclei, resulting in a polyploid nucleus which produces a nurse tissue or endosperm. The endosperm may be consumed at an early stage or persist in the seed but without this the embryo seldom comes to maturity.

Only a couple of years after the discovery of double fertilization Mendel's laws were rediscovered and this gave a great stimulus to an intelligent programme of breeding plants and animals with a view to creating superior types. Many times all that was necessary for the plant breeder was to take the usual precautions, put the pollen upon the stigma, and pray for results in the ovary. However, there were frequent difficulties not only in making wide crosses between different varieties, species and genera but also when the pollen of a flower was put upon its own stigma in certain species.

Some of the commonest difficulties are: (*a*) the inability of the pollen

grains to germinate on the stigma, (*b*) the slow growth or bursting of the pollen tube so that it does not reach the embryo sac in time, (*c*) an early abscission of the flowers before the pollen tube can approach the ovule, and (*d*) the abortion of the embryo in the ovule. There are also other problems like a lack of co-ordination between the flowering time of the male and female parents but this is often remedied by varying the planting time or regulating their day-length (photoperiod). It is also possible to store the pollen over varying lengths of time by keeping it at a low relative humidity and freezing temperatures. Some people have used even the temperatures of liquid air and stated that under such conditions many pollens can be given 'eternal life'.

So far as the initial germination of the pollen is concerned most stigmas are not too specific. Occasionally the lack of germination is due to boron deficiency which is easily corrected by spraying. Or, the stigmatic papillae have a thick cuticle which has to be dissolved by an application of the enzyme cutinase. It is even possible to wound or crush the stigma to a certain extent; or to use slightly young or old stigmas in which the power of resistance is either not fully developed or has already passed its peak. It is also practicable to cut away the original stigma and use an artificial stigma made of sugar, gelatin and other such substances.

The main trouble lies in the subsequent growth of the pollen tube in the style. This may be due to two reasons: either the maximum length attainable by the pollen tubes of the male parent is inadequate for enabling them to reach the ovules, or the path which the tubes have to traverse is unfavourable and causes the growth to slow down. One of the remedies suggested is to cut away a part of the style to a length suitable for the pollen tube and then pollinating the stub instead of the stigma. However, since the cut end may not always be as suitable for the germination of the pollen as the stigma, it is possible to remove the middle portion of the style and join the upper and lower portions by means of gelatin. Yet another method for stimulating the growth of the pollen tube is the application of certain chemical substances to the stigmas. It has been shown that auxins, plant hormones and vitamins appreciably stimulate the germination of the pollen and the rate of elongation of the tubes.

Intraovarian Pollination

Another possibility is the direct introduction of the pollen into the ovary so that the inhospitable pathway of the stigma and the style can be avoided altogether. Strasburger (1886) was perhaps the first to think of this technique of intraovarian pollination. Dahlgren (1926) succeeded in bringing about the fertilization of the ovules of *Codonopsis ovata* by cutting away the style and putting the pollen on the upper end of the ovary. Similar experiments, performed by Cappellietti (1937) on *Digitalis purpurea* and Bosio (1940)

on species of *Helleborus* and *Paeonia*, confirmed the possibilities of this technique. More recently intraovarian pollination experiments have been successfully conducted at the University of Delhi with some members of the Papaveraceae.

Since the papaver ovary contains numerous ovules and has adequate space to hold a fair quantity of fluid, five species—*Papaver rhoeas* L., *P. somniferum* L., *Eschscholzia californica* Chem., *Argemone mexicana* L. and *A. ochroleuca* Sweet—were selected for experimentation.

The first step in such experiments is the determination of the time of the opening of the flowers and of the dehiscence of the anthers. Next, the anthers are removed from a few selected flower buds before they have discharged the pollen and the buds are enclosed in cellophane bags; this is to prevent accidental pollination. Or, the stigma is smeared with collodion and thus rendered non-receptive. The third step is to find the conditions necessary for the optimum germination of the pollen under artificial conditions.

In Delhi all the five plants flower during winter. Since, in *Papaver* and *Argemone*, the anthers start dehiscing one day prior to the opening of the flowers, the flowers were emasculated three or four days earlier. In *Eschscholzia* the anthers open right on the day of anthesis, and emasculation was therefore carried out only one day earlier. In each case either the stigmas were coated with collodion or the flower buds were enclosed in cellophane bags.

In all the species the anthers dehisce in the early hours of the morning. The pollen grains were cultured by the hanging drop technique in tap water, distilled water, aqueous solutions of sucrose (0·1–2·0 m), aqueous solutions of boric acid (100 mg/l–400 mg/l), and different concentrations of sucrose (0·1–2·0 m) plus boric acid. In such cultures a single drop of the desired nutrient is placed in the centre of a cover glass and the pollen grains suspended in this drop. A cavity slide is then taken and a little vaseline applied around the cavity with a glass rod. After this the cover glass is inverted over the hollow and pressed down so that it becomes attached to the slide. From estimates made of the percentage of germination and the length of the pollen tubes, it was found that a boric acid solution of 100 mg/l gives the highest percentage of germination (40%) as well as the longest pollen tubes (990–1,000 μ). This was therefore considered as the best medium for the direct injection of the pollen grains into the ovary.

A pollen suspension was prepared in 2 ml of sterile, double distilled water with 100 mg/l of boric acid. Each drop of the suspension contained 100–300 pollen grains. The flowers, which had already been emasculated and bagged, were now used for subsequent experimentation. Surface sterilization of the ovaries was carried out by wiping them with cotton soaked in ethanol. Two punctures were made on the ovary wall—one for injecting the pollen suspension and the other on the opposite side to allow the air to

escape. The injections were given by means of a hypodermic all-glass 1 ml syringe either on the day of opening of the flower or one day after that. The syringe was shaken before each operation in order to make the pollen disperse uniformly in the fluid. Immediately after the injection, the punctures were sealed with petroleum jelly.

The experiment was conducted in five different sets. In the first or control set the flowers were allowed to become pollinated in nature. In the second set the ovary wall was pricked with the syringe but rebagged without introducing any pollen. In the third and fourth sets pollen suspensions in 100 mg/l and 200 mg/l boric acid solutions were injected as described above. In the fifth set the pollen grains were introduced as such into the ovary either through a slit made on its wall (Fig. 2K, L) or through an opening made from above after cutting away the stigma. The treated materials were fixed in acetic alcohol at intervals of three days. For detailed studies some of the ovules were dissected; others made into whole mounts; and still others sectioned with a microtome, stained with iron-haematoxylin and erythrosin, and mounted in Canada balsam.

In the naturally pollinated flowers of *Papaver* the pollen grains germinate readily on the stigma. The pollen tubes pass in between the flaps of the stigmatic rays, traverse the placental surface and reach the micropyles 24 hours after pollination (Fig. 1). The fusion of one male gamete with the nucleus of the egg and of the other with the two polar nuclei occurs three days after pollination, and in another three days the embryo sac shows a 2- to 5-celled proembryo and several endosperm nuclei. The ovules, although small and translucent on the day of anthesis, become opaque and mealy white after nine days and attain their full size in 12–15 days (Fig. 2A–D). Cell formation in the endosperm begins about the seventh day. A 15-day-old seed shows well-differentiated cotyledons, and nearly 4,000 seeds are produced per capsule. The fruit takes about a month to mature. In *Eschscholzia* and *Argemone* also the development of the fruit and seeds follows a more or less similar course.

The ovaries in the second set of experiments enlarged slightly but did not develop any seeds. This was quite expected since pollen grains were excluded from these ovaries. In *Eschscholzia* even unpollinated ovaries remained healthy for about a week but dried up soon after. About 1 per cent of them developed parthenocarpically but had no viable seeds in them.

When the suspension was injected into the ovaries, the pollen grains germinated right inside the ovarian cavity (Fig. 3G) and some of the pollen tubes entered the micropyle (Fig. 3H). Fertilization occurred as in nature in 2–4 days. The development of the embryo and endosperm compared well with that in naturally pollinated ovaries. The seeds appeared mealy white and the ovaries developed into capsules (Figs. 2E–H; 3A–F) containing viable seeds (Fig. 2I, J). However, the size of the capsule was only about two-thirds

of the normal. In fact the diameter of the capsule depended upon the number of seeds it produced. This in turn was governed by several factors, such as the age of the ovary at the time of the pollen injections, the number of pollen grains in the suspension, distribution of the pollen in the ovary, percentage of germination of the pollen, and incidence of fertilization.

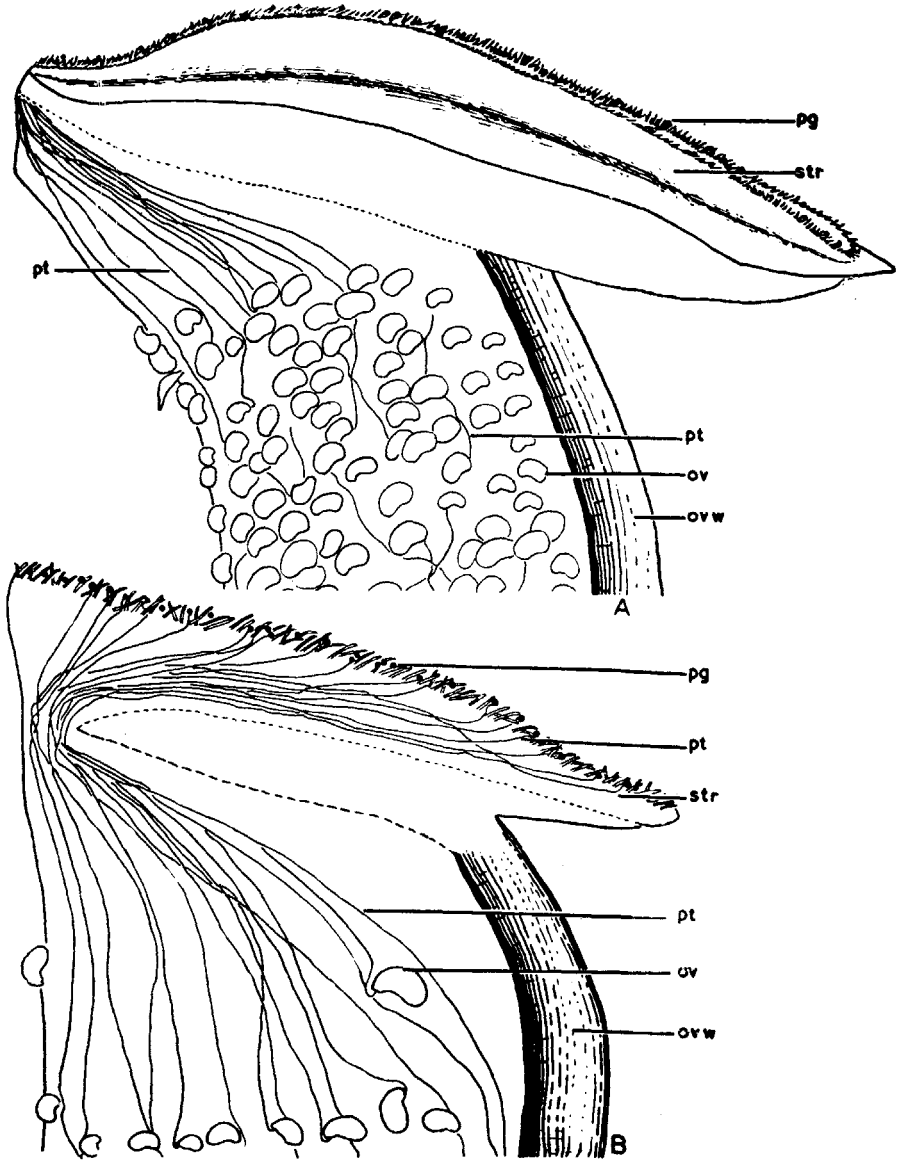

FIG. 1. *Papaver somniferum* (*ov*, ovule; *ovw*, ovary wall; *pg*, pollen grain; *pt*, pollen tube; *str*, stigmatic ray). A, B. Whole mounts of stigmatic rays and portions of placentae showing the path of the pollen tubes *in vivo*. In B the stigmatic ray has been cut open. Several tubes can be traced to the ovules.

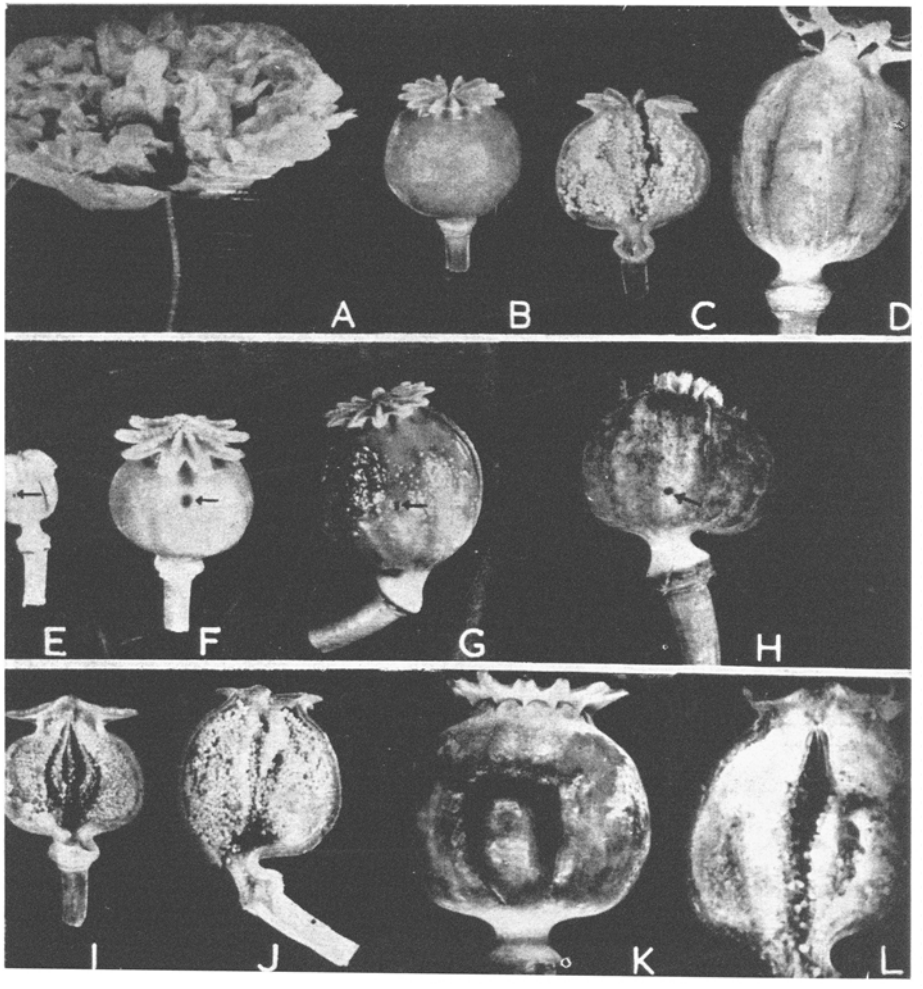

FIG. 2. *Papaver rhoeas*. A–D. Naturally pollinated field controls. A. Flower at anthesis. B, D. 9- and 40-day-old fruits. C. Vertical half of fruit shown in B. E–J. Ovaries after intraovarian pollination with pollen suspension in 100 mg/l boric acid solution. E–H. Ovaries 1, 6, 9 and 40 days after injection; the arrows indicate points of injection. I, J. Same as F, G, cut vertically. K. 21-day-old ovary with pollen inserted through a slit. L. Same, sliced vertically to show seeds. A ×0·4; B, C, E–J ×0·9; D ×1·1; K, L ×1·2.

In *Papaver somniferum* the largest number of seeds was obtained in the fifth set where the pollen grains were introduced as such into the ovary after removing the stigma. The next in the series was the third set in which a suspension of pollen in a 100 mg/l boric acid solution was injected into the ovaries. In *Eschscholzia* the best results were obtained with a pollen suspension in 200 mg/l boric acid solution. The fruit required almost the same time for maturation although the average number of viable seeds per capsule was

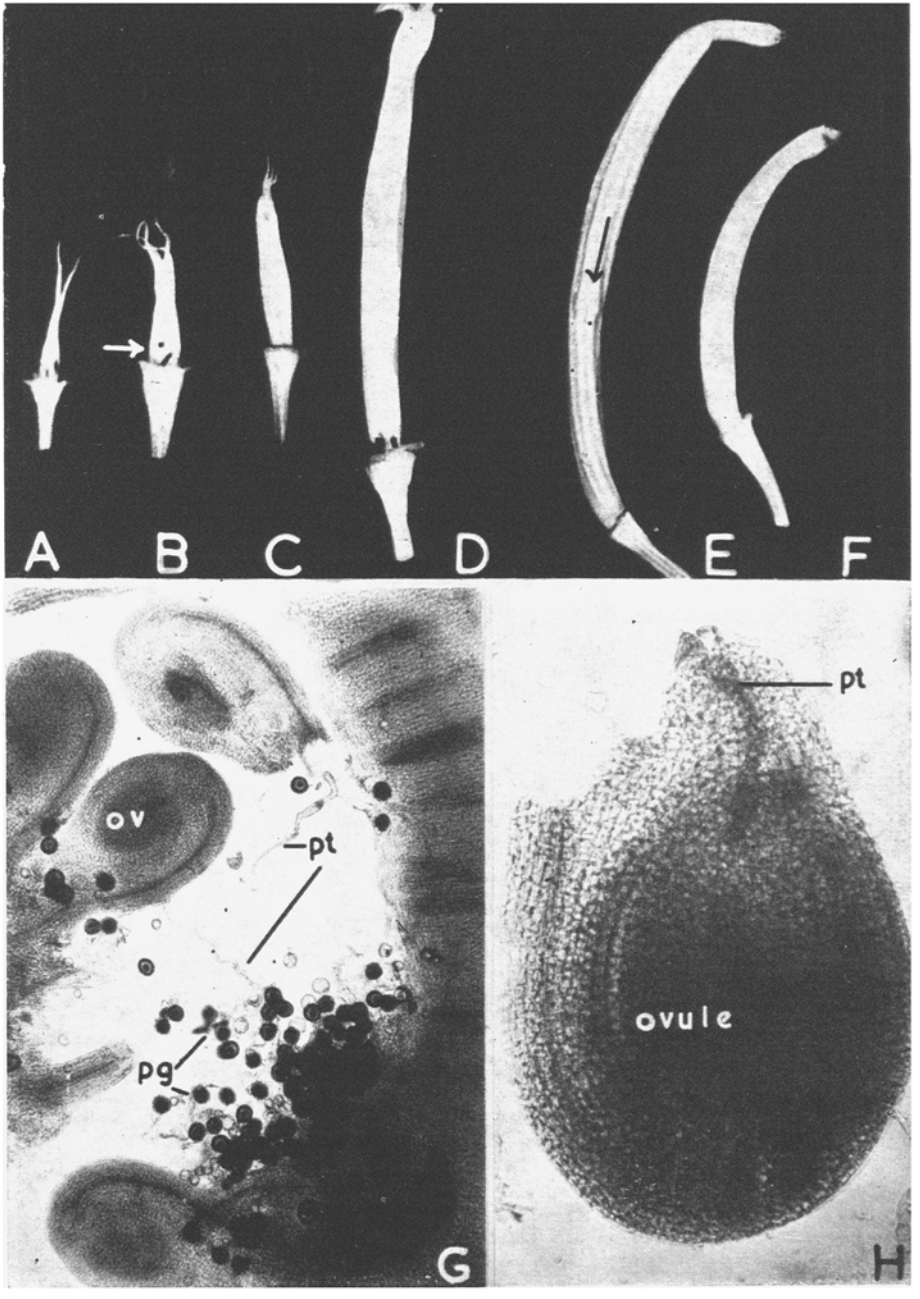

FIG. 3. *Eschscholzia californica*. Development of ovaries into fruits through injection of pollen suspension in a 200 mg/l solution of boric acid. A. Ovary on the day of injection. B–F. Ovaries 3, 6, 11, 17 and 25 days after injection. G. T.s. portion of ovary 3 days after injection, to show ovules (*ov*), pollen grains (*pg*) and pollen tubes (*pt*). H. Whole mount of ovule 4 days after injection. Note pollen tube (*pt*) in micropyle. A–F ×1·2; G ×57; H ×131.

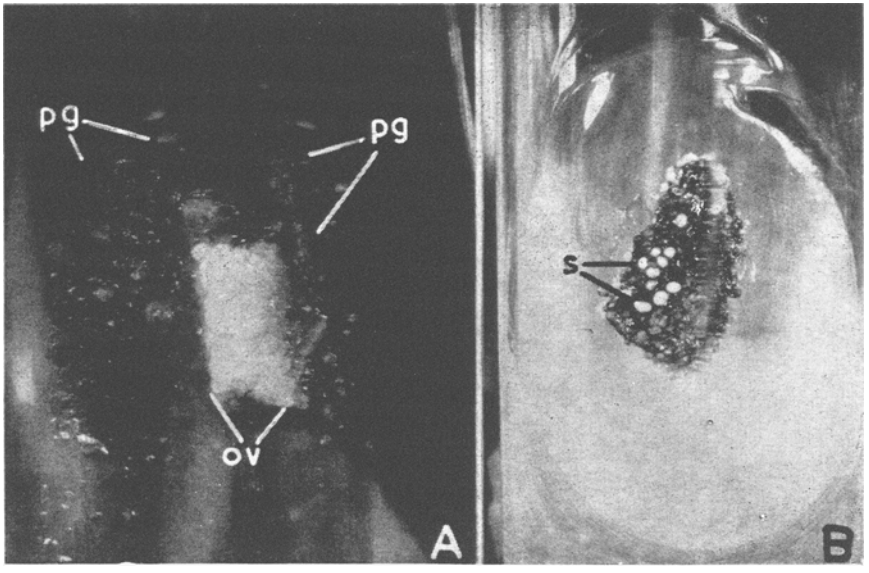

FIG. 4. *Papaver somniferum*. A. Test-tube fertilization of ovules (*ov*) attached to a portion of the placenta inoculated on Nitsch's basic medium along with pollen grains (*pg*) on the day of anthesis. ×3·5. B. An older culture showing several developing seeds (*s*). ×2.

25 as against 83 in nature. If the pollen grains were introduced as such into the ovary through a vertical slit, the average number of seeds produced was only nine per capsule. In *Argemone* no seeds could be obtained with pollen suspensions but the germination was satisfactory when the pollen grains were introduced as such into the ovarian cavity. The seeds produced were fully viable and gave rise to healthy seedlings.

These results suggest that direct injections of the pollen into the ovary may be useful when the barriers to fertilization lie in the stigma or style.

FERTILIZATION IN TEST TUBE

A newer and still more promising technique is to grow both ovules and pollen in close proximity in an artificial medium (Fig. 4A). In this method the gynoecial tissues are omitted altogether. It has been successfully accomplished in my laboratory in three members of the Papaveraceae—*Papaver somniferum*, *Eschscholzia californica* and *Argemone mexicana*; and two of the Solanaceae—*Nicotiana rustica* and *N. tabacum*. It has been designated by us as test-tube fertilization since all the stages, *i.e.* the germination of the pollen, fertilization and the formation of viable seeds, have been obtained right in the culture medium (Maheshwari and Kanta 1964).

The preliminary steps in the process are the same as in intraovarian pollination. The flower buds were emasculated at the bud stage before the dehiscence of the anthers and bagged to prevent any chance pollination.

The pollen was collected under aseptic conditions and attempts were made to devise a nutrient medium which would be suitable for the germination of the pollen grains as well as the development of the ovules. Out of several media Nitsch's nutrient medium was found to be the most effective and 10 ml of this were dispensed in each test tube and solidified in a slanting fashion. Four kinds of cultures were made. In the first set only the unpollinated ovules were grown; in the second there were some ovules along with the placentae and sometimes also a portion of the ovary wall; in the third set unpollinated ovules were cultured along with some pollen grains; and the fourth set had ovules attached to their placentae together with some pollen grains (Fig. 4A).

Before removing the ovules, the ovaries were surface-sterilized with alcohol. In *Papaver* they were simply dipped in rectified spirit and flamed. Since the ovaries of *Eschscholzia*, *Argemone* and *Nicotiana* are more delicate, they were sterilized by dipping them in chlorine water and then in alcohol. The ovules were lifted out and finally sown on the culture medium. The anthers were picked out a few hours before dehiscence and surface-sterilized in the same manner after which the pollen was scooped out with a fine scalpel and dusted over and around the ovules. Care was taken to maintain aseptic conditions throughout. Different methods were employed to study the contents of such artificially pollinated ovules. Some were dissected under the stereoscopic microscope while others were run through the alcohol-xylol-paraffin series and sectioned with the microtome.

When the ovules were excised along with the placentae from the unpollinated ovaries and cultured on Nitsch's medium without the accompaniment of pollen grains, the ovules failed to develop further. However, when pollen grains were also dusted on the culture medium, they germinated within 15 minutes. The pollen tubes covered the surface of the ovules and the placentae within two hours, and fertilization was effected in a couple of days. The incidence of fertilization was increased when the ovules were excised 24 hours after the opening of the flower. The fertilized ovules enlarged rapidly and in about a week they turned mealy white (Fig. 4B) as in nature. The early development of the embryo and endosperm was normal and matched with that in nature but subsequently the growth of the artificially fertilized ovules was quicker and often surpassed that in nature. A 22-day-old seed contained a fully formed dicotyledonous embryo and cellular endosperm. Thus all the stages—pollination, germination of pollen, double fertilization, and development of endosperm and embryo—proceeded normally in the test tube. A few ovules, although fertilized, failed to reach maturity due to an early degeneration of the embryo or the endosperm.

The mature seeds did not germinate if left on the placenta but if excised and cultured afresh in another test tube they produced healthy seedlings

within a fortnight. Thus the period of dormancy, normally shown by the seeds produced in nature, was eliminated.

Ovules sown in the culture medium without any pollen collapsed and did not develop further.

Although the test-tube seeds of *Eschscholzia* showed a normal embryo, the endosperm was poorly developed and often degenerated before becoming completely cellular. However, the seeds germinated in two or three months from the time of starting the cultures. Sometimes the hypocotyl showed an active proliferation and gave rise to a mass of undifferentiated cells or callus. On transferring the callus to a fresh medium, it produced embryo-like bodies which developed into normal seedlings. The pollen grains in *Argemone* germinated within four hours after culturing, and fertilization was effected in the usual way. The fertilized ovules contained healthy embryos and a well-developed endosperm, and one-month-old seeds were mature and black as in nature. In *Nicotiana* also the seeds obtained in test tubes gave rise to healthy seedlings when cultured on a fresh medium.

Conclusion

The technique of test-tube fertilization provides new possibilities of overcoming incompatibilities. It may prove useful in those cases where the two parents do not cross easily due to certain obstacles in the path of the pollen tube. Since the period of dormancy is also eliminated under artificial conditions, the life-cycle of a plant can be completed in a much shorter time than in nature. The next step would be to grow eggs into embryos without fertilization. The formation of such parthenogenetic embryos would facilitate the task of the breeder in producing a homozygous true-breeding type which otherwise requires a long and laborious process of selfing through several generations.

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References

Amici, G. B. (1824). Observations microscopiques sur diverses espèces de plantes. *Annls. Sci. nat. Bot.*, **2**, 41–70, 211–248.

——— (1830). Note sur le mode d'action du pollen sur le stigmate. Extrait d'une lettre d'Amici à Mirbel. *Annls. Sci. nat. Bot.*, **21**, 329–332.

Bosio, M. G. (1940). Ricerche sulla fecondazione intraovarica in *Helleborus* e *Paeonia*. *Nuovo G. bot. ital.* n.s., **47**, 591–598.

Camerarius, R. J. (1694). De sexu plantarum epistola. Tübingen.

Cappellietti, C. (1937). Ricerche preliminari sulla fecondazione intraovarica in *Digitalis purpurea* L. *Nuovo G. bot. ital.* n.s., **44**, 613.

Dahlgren, K. V. O. (1926). *Svensk bot. Tidskr.*, **20**, 97.

Guignard, L. (1899). Sur les anthérozoides et la double copulation sexuelle chez les végétaux angiospermes. *Revue gén. Bot.*, **11**, 129–135.

Hofmeister, W. (1849). Die Entstehung des Embryo der Phanerogamen. Leipzig.

Maheshwari, P., and Kanta, Kusum (1964). Control of fertilization. *In* Pollen Physiology and Fertilization, Amsterdam (Ed. H. F. Linskens), 187–193.

Nawaschin, S. G. (1898). Resultate einer Revision der Befruchtungsvorgänge bei *Lilium martagon* und *Fritillaria tenella*. *Izv. imp. Akad. Nauk*, **9**, 377–382.

Schleiden, M. J. (1837). Einige Blicke auf die Entwicklungsgeschichte des vegetablischen Organismus bei den Phanerogamen. *Arch. Bwl. Naturgesch.* III, **1**, 289–320.

Strasburger, E. (1884). Neue Untersuchungen über den Befruchtungsvorgang bei den Phanerogamen. Jena.

——— (1886). Über fremdartige Bestäubung. *Jb. wiss. Bot.*, **17**, 50–98.

Von Mohl, H. (1863). Giambattista Amici. *Bot. Ztg*, **21**, 1–8.