Chapter 10

In Vitro Pollination And Fertilization

10.1. INTRODUCTION

Sexual hybridization is a powerful tool in the hands of plant breeders for producing superior plants by combining characters distributed in different members of a species or different species of a genus. The technique involves controlled, artificial pollination of the female parent with pollen from the selected male parent. In angiosperms the female gametophyte, enclosing the egg (female gamete), is deep-seated in the ovarian cavity, well protected by the ovular tissues. The pollen grains are normally held at the stigma and there is no device for them to reach the egg. To effect fertilization the pollen grains germinate on the stigma by putting forth a tube (pollen tube) which grows through the stigma and style, finds its way into the ovule and discharges two sperms in the vicinity of the egg. One of them fuses with the egg, forming a zygote (the progenitor of the embryo), while the other fuses with the polar nuclei forming the primary endosperm nucleus.

In nature, a stigma receives a variety of pollen grains, but not all those that reach the stigma succeed in effecting fertilization. The stigma and style are equipped with devices to allow pollen of only the right mating type to function normally; others are discarded. Consequently, in hybridization programmes transferring viable pollen from one parent to the receptive stigma of another does not always lead to seed-set. Some of the barriers to fertilization are: (a) inability of pollen to germinate on foreign stigma; (b) failure of the pollen tube to reach the ovule due to excessive length of the style, or slow growth of the pollen tube, so that ovary abscises before the pollen tube reaches the base of the style; and (c) bursting of the pollen tube in the style. These are pre-fertilization or pre-zygotic barriers. In other cases fertilization may occur normally, but the hybrid embryo fails to attain maturity due to embryo-endosperm incompatibility or poor development of the endosperm (post-fertilization barriers).

From time to time various techniques have been developed to circumvent the pre-zygotic barriers to fertilization. Some of these are: (i) bud pollination; (ii) stub pollination; (iii) heat treatment of the style; (iv) irradiation; and (v) mixed pollination (see Bhojwani and Bhatnagar, 1990).

The possibility of effecting fertilization by introducing pollen grains directly into the ovary (intra-ovarian pollination) is yet another approach to bypass the pre-fertilization barriers. As early as 1926, Dahlgren (cited in Maheshwari, 1950) was able to bring about fertilization in *Codonopsis ovata* by placing pollen on the cut surface at the top of the ovary. The detailed work on developing the technique of intra-ovarian pollination was, however, done at the University of Delhi by P. Maheshwari and his associates. Kanta (1960) and Kanta and Maheshwari (1963a) obtained a good seed-set through intra-ovarian pollination in several members of the Papaveraceae (*Papaver rhoeas*, *P. somniferum*, *Eschscholtzia californica*, *Argemone mexicana*, *A. ochroleuca*). Using this technique, Kanta and Maheshwari (1963a) developed hybrids between *A. mexicana* and *A. ochroleuca*. Pollen were collected from dehiscing anthers and suspended in sterile double-distilled water containing 100 mg l^{-1} boric acid, at a density of about 100–300 grains per drop. The flowers used as the female parent were emasculated and bagged 2 days before anthesis. On the day of anthesis the ovary was surface-sterilized by wiping it with cotton soaked in ethanol, and the pollen suspension injected into the ovary through a hole using a hypodermic syringe. Another hole was made opposite the point of injection to allow the air to escape. The suspension was injected until the ovarian cavity was filled, and the liquid started to come out of the other hole. After introducing the pollen suspension the holes were sealed with petroleum jelly. Unlike *Papaver* and *Eschscholtzia*, in *Argemone* insertion of pollen through a slit proved better than the injection of suspension through a pore. While emasculating the flowers, only stamens were removed because excising the calyx and corolla led to yellowing of the ovary.

Although the technique of intra-ovarian pollination appears promising, there are no available data showing whether this technique was useful for overcoming barriers of infertility except for the cross between *Argemone mexicana* and *A. ochroleuca* (Kanta and Maheshwari, 1963a).

A still more promising and proven technique developed by Maheshwari and his students to overcome the pre-zygotic barriers to fertility is what they have described as 'test-tube fertilization'. In this technique the stigmatic, stylar and ovary wall tissues were completely removed from the path of the pollen tube, and the exposed ovules were directly dusted with pollen grains and cultured in nutrient medium until seed maturity. 'Test-tube fertilization' was first reported in *Papaver somniferum* (Kanta et al., 1962; see Fig. 10.1). The pollen grains lodged on the ovules germinated within 15 min and fertilization was effected within 1–2 days after pollination. Within 5 days the fertilized ovules enlarged, became turgid and opaque, and contained a four-celled proembryo and free nuclear en-

dosperm. Fully differentiated dicotyledonous embryo developed after 22 days. Kanta and Maheshwari (1963b) reported similar success with some other taxa belonging to the Papaveraceae (*Argemone mexicana*, *Eschscholtzia californica*) and the Solanaceae (*Nicotiana rustica*, *N. tabacum*). Since then the technique of 'test-tube fertilization' has been successfully applied to many compatible and incompatible combinations (see Table 10.1).

10.2. TERMINOLOGY

Kanta et al. (1962), Kanta and Maheshwari (1963b) and, following them, several other authors (Zenkteler, 1965, 1967, 1970; Balatkova and Tupy, 1968; Rangaswamy and Shivanna, 1969) have described the technique of seed development through in vitro pollination of exposed ovules as 'test-tube fertilization'. Seed development following stigmatic pollination of cultured whole pistils has been referred to as 'in vitro pollination'. However, in either case fertilization of the egg occurs inside the ovule by sperms delivered by the pollen tube almost in a natural fashion. Strictly speaking, the term 'test-tube fertilization' should, as in animals (Thibault, 1969), refer to in vitro fusion of excised egg and sperm cells. Therefore, in this book, in vitro application of pollen to excised ovules is referred to as 'in vitro ovular pollination', to the ovules attached to placenta as 'in vitro placental pollination' and to stigma of intact pistils as 'in vitro stigmatic pollination', under the general term of 'in vitro pollination' (Fig. 10.2). The term 'in vitro fertilization' is used only in the case of maize where fusion of isolated egg and sperm cells has been reported (Kranz and Lorz, 1990, 1993; Kranz et al., 1990, 1991).

10.3. IN VITRO POLLINATION

10.3.1. Techniques

The preliminary steps for in vitro pollination and intra-ovarian pollination are the same, viz (a) determination of the time of anthesis, dehiscence of anthers, pollination, entry of pollen tube into the ovules, and fertilization, (b) emasculation and bagging of flower buds, and (c) collection of pollen grains. In addition, for in vitro pollination a suitable nutrient medium is required that will favour the germination of pollen grains and, more importantly, the development of fertilized ovules into mature seeds.

A principal requirement for in vitro pollination is the maintenance of reasonable sterility during pollen and ovule collection. To prevent chance

pollination the buds to be used as the female partner are emasculated before anthesis and bagged. One or two days after anthesis the buds are brought to the laboratory and prepared for aseptic culture. If necessary, sepals and petals are removed and the pistil along with the pedicel, if present, is given a quick rinse in 70% alcohol, surface-sterilized with a suitable agent (see Chapter 2) and finally washed thoroughly with sterile distilled water. The stigma and style are removed and the ovary wall is peeled to expose the ovules. The whole placenta bearing the ovules and attached to a short pedicel is generally used for placental pollination. Alternatively, the placenta may be cut into two or more pieces each carrying a certain number of ovules. Balatkova and Tupy (1968, 1972, 1973) split the placenta carrying the ovules into two longitudinal halves and planted them individually with their cut ends in contact with the medium. Rangaswamy and Shivanna (1971b) further modified the technique of placental pollination. They cultured the entire pistils and exposed the placenta bearing the ovules by removing the ovary wall. Such a system would allow a study of the effect of simultaneous placental and stigmatic pollination in the same pistil (see also Wagner and Hess, 1973; Hess and Wagner, 1974). To perform in vitro stigmatic pollination the excised pistils are carefully surface-sterilized without wetting the stigma with the sterilant solution.

For collecting the pollen under aseptic conditions undehisced anthers are removed from buds and kept in sterile petri plates until they dehisce. When anthers are to be taken from open flowers they may be surface-sterilized and left in sterile petri plates containing a pre-sterilized filter paper until their dehiscence. The discharged pollen is aseptically deposited on the cultured ovules, placenta, or stigma, as the case may be. It has been reported that the pollen deposited on ovules or placenta perform better than those spread on the medium around the ovules. Free water on the surface of ovules may also inhibit pollen germination. Therefore, the film of water on the ovules should be removed with the point of a filter paper (Zenkteler et al., 1987).

Fig. 10.1. In vitro pollination in Papaveraceae. (A,B,D,F–H, *Papaver somniferum*; C, *Eschscholtzia californica*; E, *Argemone mexicana*). (A) Portion of placenta with ovules pollinated in vitro. (B) Whole mount from pollinated culture, showing pollen germination and pollen tube growth on and around the ovule. (C) Whole mount from 1-day-old pollinated culture, showing pollen tube entry into the ovule. (D) Seven-day-old culture; several young seeds are seen on the placenta. (E,F) Proembryo and globular embryo, respectively, excised from seeds developed in vitro. (G) Longisection of seed 15 DAP, showing normal embryo and endosperm. (H) Germinated seeds excised from 22-day-old pollinated culture (A,C,E,G and H after Kanta and Maheshwari, 1963b; B,D and F after Kanta et al., 1962).

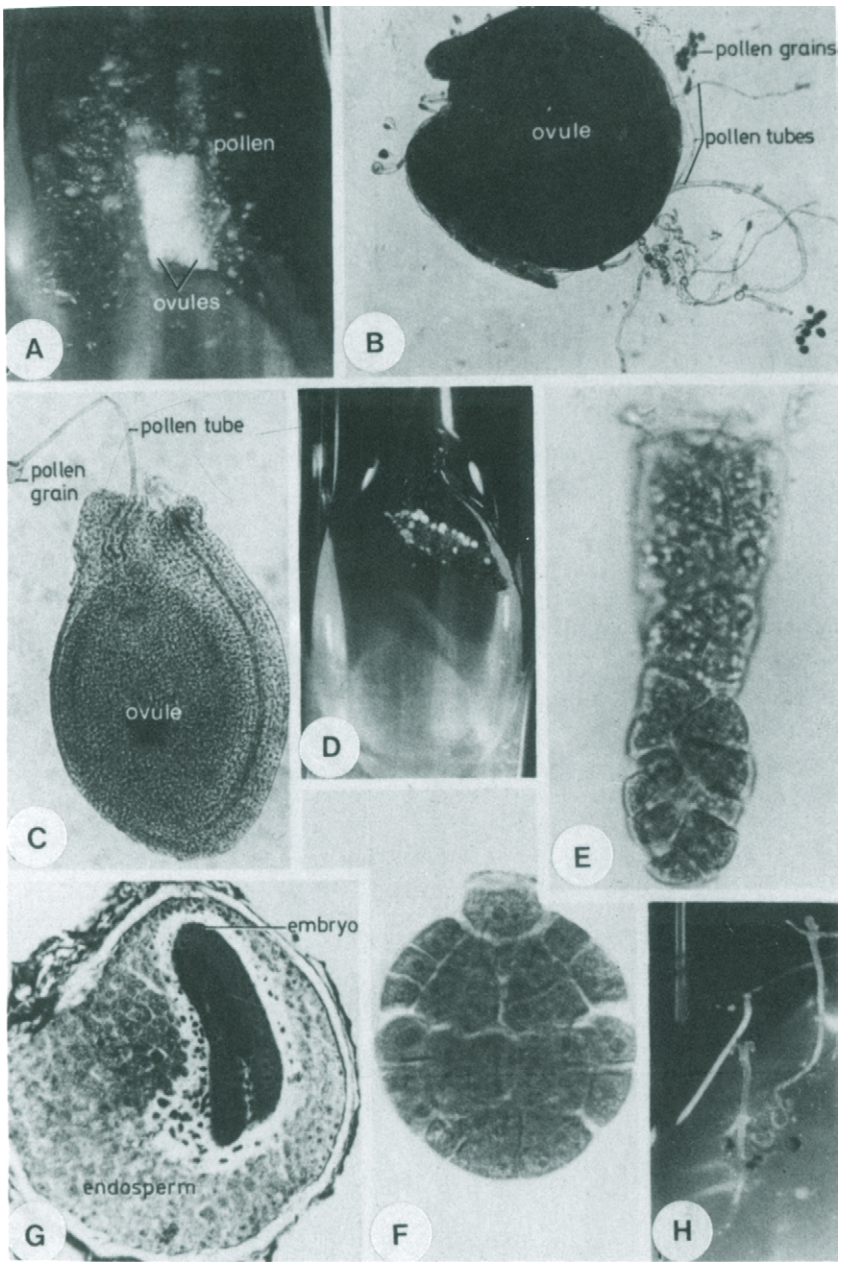
pollen
ovules
A
ovule
pollen grains
pollen tubes
B
pollen tube
pollen grain
ovule
C
D
E
embryo
endosperm
G
F
H

TABLE 10.1

Examples where viable seeds were produced after in vitro ovular or placental pollination

Species	Medium[a,b]			Reference
	Basal medium	Sucrose conc. (%)	Supplements (mg l^{-1})	
Self-pollination				
Agrostemma githago	N	5	CH (500)	Zubkova and Sladky (1975)
Argemone mexicana	N	5	CH (500)	Kanta and Maheshwari (1963b)
Brassica campestris	MS	2	–	Zenkteler et al. (1987)
B. napus	MS	2	–	Zenkteler et al. (1987)
B. oleracea	N			Kameya et al. (1966)
Dianthus caryophyllus	W	4	–	Zenkteler (1965)
Dicranostigma franchetianum	N	4	–	Rangaswamy and Shivanna (1969)
Digitalis purpurea				Zenkteler and Slusarkiewicz-Jarzina (1986)
D. lutea				Zenkteler and Slusarkiewicz-Jarzina (1986)
Diplotaxis tenuifolia				Zenkteler (1988)
Eschscholtzia californica	N	5	CH (500)	Kanta and Maheshwari (1963b)
Glycine max	MS,B_5	6		Tilton and Russell (1983, 1984)
Melandrium album	W,N	2,5	–, CH (500)	Zenkteler (1967); Zubkova and Sladky (1975)
M. rubrum	W	2	–	Zenkteler (1967)
Nicotiana alata	LS			Zenkteler (1980)
N. knightiana				Zenkteler and Slusarkiewicz-Jarzina (1986)
N. longiflora				Zenkteler and Slusarkiewicz-Jarzina (1986)
N. rustica	N	5	CH (500)	Kanta and Maheshwari (1963b)
N. tabacum	N	5	CH (500)	Kanta and Maheshwari (1963b)
Papaver nudicaule	N	5	CH (500)	Olson and Cass (1981)
P. somniferum	N	5	CH (500)	Kanta et al. (1962)
Petunia axillaris	RS	4	–	Rangaswamy and Shivanna (1971a,b)

P. hybrida	N	4	CH (500)+ IAA (0.1)	Wagner and Hess (1973)
P. parodii				Zenkteler and Slusarkiewicz-Jarzina (1986)
Primula pubescence				Zenkteler and Slusarkiewicz-Jarzina (1986)
Scopolia carniolica				Zenkteler and Slusarkiewicz-Jarzina (1986)
Sisymbrium loescli				Zenkteler (1988)
Torenia fournieri				Zenkteler and Slusarkiewicz-Jarzina (1986)
Trifolium repens	MS	3	CH (100)	Leduc et al. (1992)
Zea mays	W,N,MS	17	Yolk of hen's egg (100 drops l^{-1})	Sladky and Havel (1976)
	LS	15		Gengenbach (1977a,b)
	GP	5	GA_3 (10)	Dhaliwal and King (1978)
	MS	7	CH (500)+ IAA (1)+ Kn (0.5)	Bajaj (1979)
	MS	5	GA_3 (10.4)	Raman et al. (1980)
	MS	5	CH (500)	Dupuis and Dumas (1990)
Cross-pollination				
Brassica napus × *B. campestris*	MS	2		Zenkteler et al. (1987)
B. oleracea × *B. cretica*				Zenkteler (1992)
B. oleracea × *Diplotaxis tenuifolia*				Zenkteler (1992)
Melandrium album × *M. rubrum*	W	2	–	Zenkteler (1967)
M. album × *Viscaria vulgaris*	W	2	–	Zenkteler (1969)
M. album × *Silene schafta*	W	2	–	Zenkteler (1967, 1969)
M. rubrum × *M. album*	N,W	2	–	Zenkteler (1969)
Nicotiana alata × *N. debney*				Zenkteler (1992)
N. tabacum × *N. amplexicaulis*	N	4	CH (500)	DeVerna et al. (1987)
N. tabacum × *N. benthamiana*	N	4	CH (500)	DeVerna et al. (1987)
N. tabacum × *N. debney*	N,MS	2	–	Zenkteler (1980)
N. tabacum × *N. rependa*	N	4	CH (500)	DeVerna et al. (1987)

TABLE 10.1 (*continued*)

Species	Medium[a,b]			Reference
	Basal medium	Sucrose conc. (%)	Supplements (mg l^{-1})	
N. tabacum × *N. rustica*	N	5		Marubashi and Nakajima (1985)
Petunia parodii × *P. inflata*	W	4	–	Sink et al. (1978)
Zea mays × *Z. mexicana*	GP	5	GA_3 (10)	Dhaliwal and King (1978)

[a]GP, Green and Phillips (1975); LS, Linsmaier and Skoog (1965); MS, Murashige and Skoog (1962); N, Nitsch (1951); RS, Rangaswamy and Shivanna (1971a); W, White's medium as modified by Rangaswamy (1961).
[b]Blank spaces in columns 2–4 indicate that the information was not available.

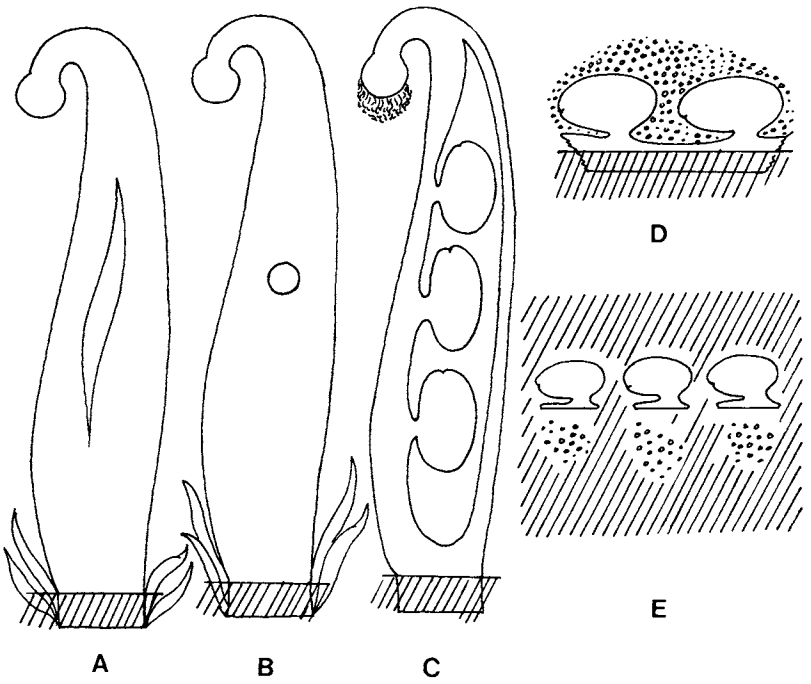

Fig. 10.2. Diagrams depicting various types of in vitro pollinations. (A,B) Intra-ovarian pollination through a slit and a pore in the ovary wall, respectively. (C) Stigmatic pollination. (D) Placental pollination. (E) Ovular pollination (modified from Tilton and Russell, 1984).

In maize, where ovaries are well protected by several layers of husks, surface sterilization has proved unnecessary (Gengenbach, 1977a, 1984). Bagged spadices are collected 2–6 days after the emergence of silk. The silks projecting from the husks are severed with a scalpel, and the outer husks removed by hand. The removal of the innermost husk and all subsequent operations are performed with sterilized instruments under aseptic conditions. Pieces of cob, each carrying a total of 4–10 ovaries in two rows, are placed on the medium in petri plates in such a way that their silks remain hanging out of the plate after putting on the lid. The silks can be pollinated with pollen not necessarily aseptic. Twenty-four hours after pollination the silks are clipped off and the petri plates sealed. Successful in vitro pollination in maize has been achieved with as few as only one pollen per silk. Generally, the fertilization rates are lower with single pollen pollination than following mass pollination (Raman et al., 1980; Hauptli and Williams, 1988) but Kranz and Lorz (1990) and Kranz and Brown (1992) reported similar fertilization rates with mass (41%) and single grain (39%) pollinations.

The most critical step of the in vitro pollination technique is the development of viable seeds from ovules (in ovular and placental pollination)

and ovaries (in stigmatic pollination) following fertilization. It would not be out of place, therefore, to discuss briefly the studies on ovule and ovary culture before dealing with the factors affecting seed-set following in vitro pollination.

(i) *Ovule culture.* Ovule culture is important not only with reference to in vitro pollination, but also because it serves as an experimental system to study the in vitro response of zygotes and very young proembryos, which are difficult to excise and culture (see Chapter 11). The first attempt to isolate ovules and culture them under aseptic conditions was made by White (1932) in *Antirrhinum majus*. However, the technique of ovule culture was developed and perfected at the University of Delhi.

In vitro development of viable seeds from ovules containing globular or older embryos is comparatively easy (*Gynandropsis* and *Impatiens*, Chopra and Sabharwal, 1963; *Nicotiana tabacum*, Dulieu, 1966; *Allium cepa*, Guha and Johri, 1966). With reference to in vitro pollination, however, it is not only desirable but absolutely essential to be able to grow very young ovules excised soon after fertilization. The first report of a successful culture of ovules containing zygote was published by Maheshwari (1958) in *Papaver rhoeas*. Maheshwari (1958) and Maheshwari and Lal (1961b) raised viable seeds of *Papaver somniferum* starting with ovules excised 6 days after pollination (DAP), when they contained a zygote or a two-celled proembryo and a few endosperm nuclei. On a rather simple medium, containing Nitsch's (1951) mineral salts, White's (1943) vitamins, and sucrose (5%) but without any growth substance, the growth of the embryo was initially slower than that in vivo but after the globular stage the embryo grew very rapidly. In 20-day-old cultures the embryos measured 0.93 mm compared to the maximum length of 0.65 mm attained by the embryos in vivo. The addition of kinetin or casein hydrolysate accelerated the initial growth of the embryo.

Poddubnaya-Arnoldi (1959, 1960) successfully grew the ovules from pollinated ovaries of several orchids simply on 10% sucrose solution. She was able to trace events from the time of the entry of the pollen tube into the ovule up to the development of the embryo.

Unlike *Papaver* and orchids, when the ovules of *Zephyranthes* containing a zygote and the primary endosperm nucleus were cultured on Nitsch's medium alone, the embryo grew up to the globular stage but failed to differentiate further (Sachar and Kapoor, 1959). The addition of growth regulators, such as kinetin, IAA, or GA_3, did not promote further growth of the embryo. However, if coconut milk or casamino acids were added to the medium the embryo developed normally and the seeds germinated in situ

(Kapoor, 1959). Individually, histidine, arginine or leucine were as effective as casamino acids. In *Trifolium repens* ovules cultured at the zygote or two-celled proembryo stage (1–2 DAP) developed into mature seeds only if the medium was supplemented with the juice prepared from young fruits (10 days after anthesis) of cucumber or watermelon (Nakajima et al., 1969). The addition of GA_3 (10 mg l^{-1}) in the presence of cucumber juice further promoted seed development from these very young ovules. Wakizuka and Nakajima (1974) noted that cucumber juice (5%) was also essential to raise fully developed, germinable seeds from *Petunia hybrida* ovules excised shortly after the entry of the pollen tube (2 DAP). Osmolarity of the culture medium has been reported to play an important role in the development of excised ovules. It is especially critical for very young ovules. In *Petunia hybrida* ovules isolated 7 DAP, enclosing a globular embryo, developed into mature seeds on a medium containing sucrose anywhere in the range of 4–10%. The optimal level of sucrose for ovules having zygote and a few endosperm nuclei was 6%, whereas for ovules just after fertilization it was 8% (Wakizuka and Nakajima, 1974).

In contrast to the reports of Maheshwari (1958) and Maheshwari and Lal (1961b), Pontovich and Sveshnikova (1966) observed that in the cultures of excised ovules (enclosing zygote or two-celled proembryo) of poppy the embryo failed to undergo differentiation even if the medium was supplemented with casein hydrolysate, coconut milk, adenine or kinetin. However, if the ovules were cultured attached to the placenta the zygote developed into a normally differentiated embryo. The beneficial effect of placental tissue on the growth and development of cultured ovules has also been observed in *Gynandropsis* (Chopra and Sabharwal, 1963). In their studies on in vitro pollination, Kanta et al. (1962) and Rangaswamy and Shivanna (1971a) recorded a similar promotive effect of placenta on seed development.

Ovule culture has proved to be a very useful technique to raise interspecific (Przywara et al., 1989; Hossain et al., 1990; Espinasse et al., 1991) and intergeneric (Hossain et al., 1988; Takahata, 1990; Ahmad and Comeau, 1991) hybrids which normally fail due to the abortion of embryo at a rather early stage when its excision and/or culture is either very tedious or impossible. The loss of a hybrid embryo due to premature abscission of fruits, as happens in many interspecific crosses in cotton, could be prevented by ovule culture (Steward and Hsu, 1978).

In the cross *Gossypium arboreum* × *G. hirsutum* the hybrid embryo develops only up to 8–10 DAP. Subsequently, numerous abnormalities occur leading to the failure of further embryo development. Beasley (1940) and Weaver (1958) cultured the immature embryos to raise full plants, but the excised embryos failed to grow. Pundir (1967) excised the

TABLE 10.2

The composition of nutrient medium for culturing young, fertilized ovules of cotton following intra- and interspecific pollination[a]

Constituents	Amount (mg l^{-1})
KNO_3	5055
NH_4NO_3	1200
$MgSO_4 \cdot 7H_2O$	493
$CaCl_2 \cdot 2H_2O$	441
KH_2PO_4	272
$FeSO_4 \cdot 7H_2O$	8.3
Na_2·EDTA	11
H_3BO_3	6.18
$MnSO_4 \cdot 4H_2O$	16.9
$ZnSO_4 \cdot 7H_2O$	8.6
KI	0.83
$NaMoO_4 \cdot 2H_2O$	0.24
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.024
Nicotinic acid	0.49
Pyridoxine·HCl	0.82
Thiamine·HCl	1.35
Inositol	180
D-Fructose	3600
Sucrose	40000
IAA	5×10^{-5} M

[a]After Steward and Hsu (1978).

ovules 3 days after cross pollination when they contained zygote or a three-celled proembryo and cultured them on Murashige and Skoog's medium containing 50 mg l^{-1} of inositol. By the 5th week in culture fully differentiated embryos were formed and by the 7th week 70–80% ovules developed hybrid seedlings. Following a similar technique, Steward and Hsu (1978) raised four different interspecific hybrids in the genus *Gossypium* which were otherwise unknown. For the composition of the medium used, see Table 10.2.

The cross *Trifolium repens* × *T. hybridum* has not been successful by the conventional or embryo rescue method. However, excising the ovules 12–14 DAP and culturing them on a medium containing 15% cucumber juice for 5–6 days provided culturable embryos (Przywara et al., 1989). Similarly, Espinasse et al. (1991) succeeded in obtaining some hybrid plants from the crosses *Helianthus annuus* × *H. maximiliani* and *H. annuus* × *H. tuberosum* by a combination of in ovulo embryo culture, for

1 week, followed by excised embryo culture. Direct embryo culture was not successful in these crosses showing post-fertilization incompatibility.

Excised ovule culture has been used by several scientists to investigate the development of cotton fibre (Beasley and Ting, 1973; Waterkeyn et al., 1975; Haigler et al., 1991). Beasley and Ting (1973) conducted detailed studies on the physiology of fibre cell elongation in cultured ovules of *Gossypium hirsutum*. The ovules excised on the day of anthesis or a day later either failed to develop or formed only a few fibres. However, most of the ovules cultured 2 days after anthesis showed the development of fibres. The ovules floating on the surface of a liquid medium produced more fibres than those which were submerged. Fibre development was better at 30°C than at 20 or 25°C. GA_3 induced marked elongation of the fibre, whereas kinetin and ABA exercised a marked inhibition. Haigler et al. (1991) found cultured ovules to be a model system to study fibre development under low temperature. Singletary and Below (1989) used ovule culture to study the effect of carbon and nitrogen supply on carbohydrate and protein accumulation in developing kernels of maize.

(ii) *Ovary culture.* LaRue (1942) was probably the first to raise aseptic cultures of angiosperm flowers. He obtained rooting of the pedicel and a limited growth of the ovaries. The technique of ovary culture was developed by Nitsch (1951) who grew detached ovaries of *Cucumis anguria*, *Fragaria* sp., *Lycopersicon esculentum*, *Nicotiana tabacum* and *Phaseolus vulgaris* on synthetic medium. The ovaries of *Cucumis* and *Lycopersicon* excised from pollinated flowers developed into mature fruits containing viable seeds. However, the fruits were smaller than those formed in nature. Similarly, the fruits of *Linaria macroccana* (Sachar and Baldev, 1958) and *Tropaeolum majus* (Sachar and Kanta, 1958) developed in vitro failed to attain a size comparable to that of fruits developed under natural conditions.

Maheshwari and Lal (1958, 1961a) succeeded in rearing the ovaries of *Iberis amara* excised from flowers 1 DAP. At this stage the ovules contained a zygote and a few free endosperm nuclei. On a simple medium containing mineral salts and sucrose, the growth of ovaries was good, but the embryo remained smaller than those formed in nature. With the addition of B-vitamins to the above medium, normal healthy fruits matching the natural fruits were obtained. In vitro formed fruits were even larger than the in vivo formed fruits when IAA was added to the mineral salts-sugar-vitamin medium (Maheshwari and Lal, 1958). In cultures, normal development of ovaries, excised at the zygote or two-celled proembryo stage, was also achieved with *Anethum graveolens* (Johri and Sehgal, 1966) and *Hyoscyamus niger* (Bajaj, 1966b). On a medium sup-

plemented with coconut milk the ovaries of *Anethum* surpassed the size of natural fruits.

In vitro studies have revealed that the floral envelopes do not function merely as protective structures for sex organs, but play an important role in the development of the fruit and the embryo. In the cultures of *Triticum aestivum* and *T. spelta* ovaries, excised soon after pollination, the embryo developed normally only if the floret envelopes (lemma and palea) were left intact. In their absence the growth of the proembryo was impaired (Redei and Redei, 1955a,b). La Croix et al. (1962) reported similar observations with barley and concluded that the 'Hull factor' is necessary for a proper development of the embryo. In the absence of the 'Hull factor' the cells of barley embryo underwent elongation and DNA synthesis but did not divide. In dicotyledonous plants removing the calyx also results in poor fruit development (Chopra, 1958, 1962; Maheshwari and Lal, 1958, 1961a; Bajaj, 1966; Guha and Johri, 1966; Richards and Rupert, 1980). It is, therefore, important that the perianth should be left intact while attempting in vitro stigmatic pollination.

Inomata (1977, 1979) raised interspecific hybrids between sexually incompatible parents *Brassica campestris* and *B. oleracea* through ovary culture. To date several other interspecific and intergeneric hybrids in the family Brassicaceae have been produced with the aid of ovary culture (Sarla and Raut, 1988; Batra et al., 1990).

10.3.2. Factors affecting seed-set following in vitro pollination

(i) *Explant.* In *Petunia axillaris* in vitro pollinated excised ovules or a group of ovules attached to a piece of placenta did not form viable seeds. The pollen grains germinated normally, but the pollen tubes failed to enter the ovules (Rangaswamy and Shivanna, 1971a). However, when intact placentae with undisturbed ovules were pollinated, normal events from pollen germination to the development of viable seeds occurred (see Fig. 10.3). Wagner and Hess (1973) reported that in *Petunia hybrida* complete removal of the style had a deleterious effect on seed-set following placental pollination. Consequently, for in vitro pollination they (Wagner and Hess, 1973; Hess and Wagner, 1974) cultured the entire pistil and exposed the ovules by simply peeling the ovary wall. In such explants if placental pollination and stigmatic pollination are made on the same pistil, the latter exhibits better fertilization.

In maize the ovaries attached to cob tissue give better results than single ovaries (Sladky and Havel, 1976; Gengenbach, 1977a; Dhaliwal and King, 1978). Reducing the number of ovaries per explant does not affect fertilization but it has a deleterious effect on kernel development

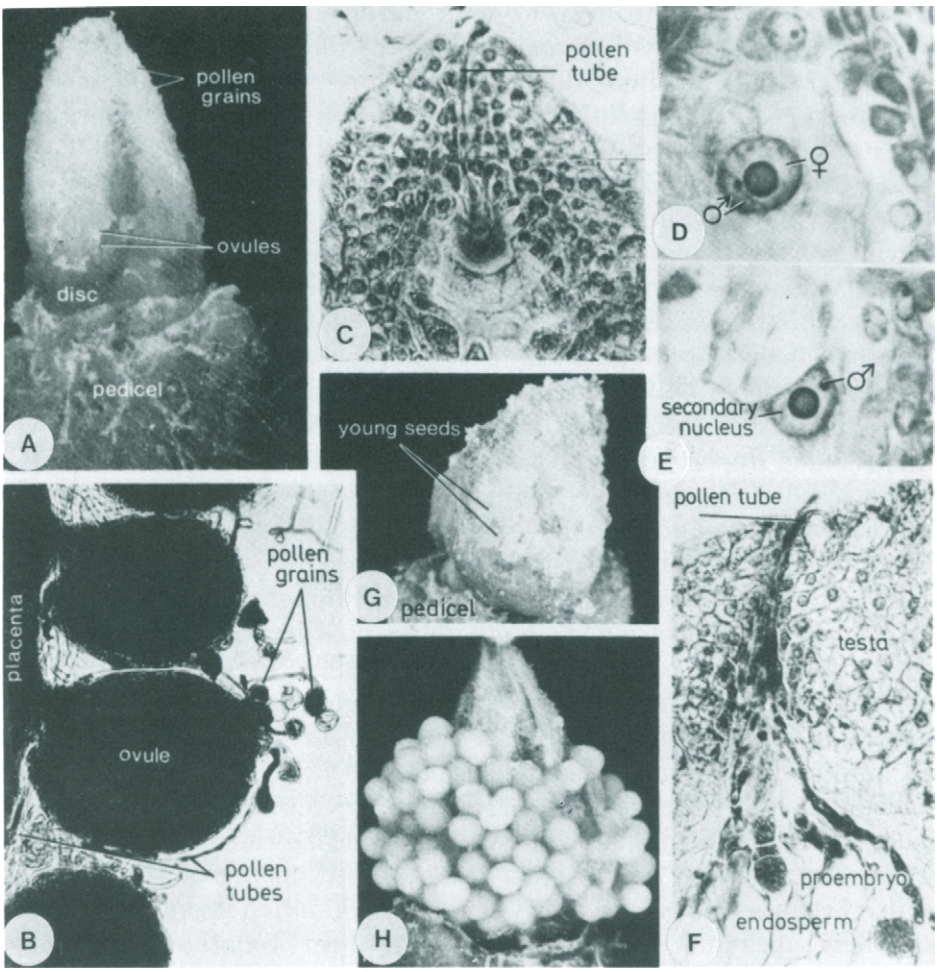

Fig. 10.3. In vitro placental pollination to overcome self-incompatibility in *Petunia axillaris*. (A) Both the placentae of an ovary, with their entire mass of ovules covered with pollen, ready for culture. (B) Free-hand section of placentae 24 h after self-pollination; the picture shows a portion of a placenta bearing 3–4 ovules. Pollen grains have germinated and the pollen tubes are seen all over and in between the ovules. (C) Longisection through micropylar region of ovule 24 h after self-pollination; note the entry of pollen tube into the embryo sac. (D,E) Sections of ovules 2 days after selfing, showing syngamy and triple fusion, respectively. (F) Micropylar end of a 7-day-old seed in longisection; note extra-micropylar part of pollen tube, remnants of pollen tube in micropyle, testa, filamentous proembryo and cellular endosperm. (G,H) Five- and 24-day-old cultures, respectively; numerous mature seeds are formed in (H). (A,B,F–H after Rangaswamy and Shivanna, 1967; C–E after Rangaswamy and Shivanna, 1971a).

(Gengenbach, 1977a; Higgins and Petolino, 1988). Gengenbach (1977a) reported that cob pieces with one or two ovaries did not form any fully developed kernel. Four ovary blocks developed only small kernels, whereas 10 ovary blocks had one or two fully developed kernels of large size. Higgins and Petolino (1988) also observed that the development of complete kernels was greatly influenced by the size of the explant and the ratio of ovule-to-cob tissue. According to these authors cob pieces bearing 30 ovaries produced twice as many fully formed kernels as with 10 ovules. Similarly, an ovule-to-cob tissue ratio of 4:24 (cob pieces with 24 ovaries were taken but only 4 ovaries per piece were pollinated; all other ovaries were removed) gave best results; depending on the genotype, 19.4–36.2% ovaries formed complete kernels.

The beneficial effect of parental tissue on ovary and ovule growth is discussed in Section 10.3.1. Role of accessory organs in seed development is also highlighted in the studies on in vitro pollination of *Trifolium* species (Richards and Rupert, 1980; Leduc et al., 1992). Besides the excision of calyx and corolla lobes, excessive injury to the ovary wall was detrimental to in vitro development of seeds. In *T. repens* even an incision along the entire length of the ovary wall, to expose the ovules for pollination, caused necrosis of the ovary. However, insertion of pollen through a pore in the ovary wall ('in vitro intra-ovarian pollination') resulted in fertilization and the formation of torpedo embryos (Leduc et al., 1992). Similarly, in *Brassica* species removal of the entire ovary wall was highly detrimental to seed-set following in vitro pollination whereas removal of only a part of the ovary wall gave the best results (Zenkteler et al., 1987). It is, therefore, recommended that in in vitro pollination studies a bare minimum of the parental tissue, necessary to pollinate the ovules, should be removed. Wetting the surface of the ovules and stigma (in stigmatic pollination) should be avoided because it may lead to poor pollen germination and/or bursting of the pollen tubes and, consequently, poor seed-set (Balatkova and Tupy, 1968; Zenkteler, 1980).

Time of excising the ovules from pistils has a definite influence on seed-set following in vitro pollination. The incidence of seed-set is higher when the ovules are excised 1–2 days after anthesis than on the day of anthesis (Kanta et al., 1962; Kanta and Maheshwari, 1963b; Balatkova et al., 1977b). The optimal stage of maize spike for in vitro pollination is 3–4 days after silking (Gengenbach, 1977b). The physiological state of the pistil at the time of excising the ovules or pistil may also influence seed-set following in vitro pollination. Balatkova et al. (1977a) observed that the unfertilized ovules of tobacco excised from the pistils pollinated with its own pollen or those of *Malus* sp. and pollinated in vitro with the pollen of tobacco gave a comparatively better seed-set than the ovules from

unpollinated pistils. Pollen germination on the stigma and pollen tube growth through the style is known to influence metabolic activities in the ovary (White, 1907; Tupy, 1961; Deurenberg, 1976). Johri and Maheshwari (1966), Sturani (1966) and Deurenberg (1976) demonstrated that pollen tube-style interaction stimulates protein synthesis in the ovary. Balatkova et al. (1977a) confirmed this observation in tobacco. These results suggest that if detailed temporal studies on pollination, pollen tube growth, entry of the pollen tube into the ovary and double fertilization are made under the conditions in which the experimental plants are grown, it should be possible to improve the chances of success of in vitro pollination by excising the ovules from pollinated pistils, but before the entry of pollen tubes into the ovary. In this context, it is interesting that in *Petunia axillaris*, a self-incompatible species, self-stigmatic pollination did not interfere with the processes of fertilization and seed development as a result of self-placental pollination simultaneously made on the same pistil (Rangaswamy and Shivanna, 1971b).

(ii) *Culture medium.* The technique of in vitro pollination involves two major processes: (a) germination of pollen grains and pollen tube growth leading to fertilization, and (b) development of the fertilized ovules into mature seeds with a viable embryo. The efficiency of the technique, to a large extent, depends on the composition of the medium which can support both processes. It is not very difficult to achieve a satisfactory germination of pollen grains and pollen tube growth. Moreover, in placental pollination, which is the most promising of the various types of in vitro pollination, pollen grains are sprinkled on the ovules where they germinate, and pollen tubes enter the ovules without even coming in contact with the medium. Where pollen grains fail to germinate on the surface of the ovules they may be separately grown on a suitable medium and pollen tubes applied to the ovules (Balatkova and Tupy, 1968). Seed development following in vitro pollination by germinated pollen has also been reported in maize (Raman et al., 1980).

Pollen grains in the Brassicaceae, which are shed at the three-celled stage, are difficult to germinate in vitro and, consequently, in vitro pollination in this group of plants is also refractory. Kameya et al. (1966) modified the technique of in vitro pollination to obtain germinable seeds in *Brassica oleracea*. They dipped the isolated ovules in a 1% solution of $CaCl_2$, planted them on a slide pre-coated with 10% gelatin solution of about 40 μm thickness and immediately pollinated them with the pollen from freshly opened flowers. The slide was stored in a covered petri plate with a moist filter paper sticking to the lid. After 24 h only the fertilized ovules were transferred to Nitsch's agar medium. Following this method,

Kameya et al. could obtain two germinable seeds from 75 ovules initially pollinated. Seed development did not occur without the calcium treatment. Promotion of pollen germination and pollen tube growth by Ca^{2+} ions is well known (see Bhojwani and Bhatnagar, 1992).

The most important role of culture medium is in supporting normal development of the fertilized ovules. It is, therefore, imperative that before attempting in vitro pollination the optimal nutritional and hormonal requirements and physical conditions for the successful culture of very young ovules (containing the zygote or a few celled proembryo) of the plant to be used as the female parent are investigated. This would improve the chances of success. Indeed, this was the secret of the first success of Kanta et al. (1962) in obtaining viable seeds through in vitro placental pollination in *Papaver rhoeas*. They used exactly the same medium as that described by Maheshwari and Lal (1961b) for the successful culture of fertilized ovules of this taxon taken 6 days after in vivo pollination.

The salt mixture commonly used for in vitro pollination is that developed by Nitsch (1951) for ovary culture. To this are added sucrose and vitamins (White, 1943). The composition of the modified Nitsch's medium widely employed to culture in vitro pollinated ovules is given in Table 10.3. Sladky and Havel (1976), who tested different basal media (White, 1943; Murashige and Skoog, 1962; Nitsch, 1969), did not find a significant difference in the response of the in vitro pollinated ovaries.

Generally, sucrose has been used at a concentration of 4–5%. Even in maize, where some authors have used as much as 15–17% sucrose (Sladky and Havel, 1976; Gengenbach, 1977a,b), Dhaliwal and King (1978) obtained viable seeds following intra- and interspecific in vitro pollination with the normal level of 5% sucrose.

The information on the effect of various growth regulators and other supplements to the basal medium on seed development from cultured ovules is very meagre. This seems to be the reason for the failure of seedsets especially when pollen germination and pollen tube entry into the ovule (Niemirowicz-Szczytt and Wyszogrodzka, 1977) and fertilization (Zenkteler and Melchers, 1978) in vitro occurred normally. Generally, the basal medium has been supplemented with 500 mg l^{-1} of casein hydrolysate (CH; see Table 10.1). Rangaswamy and Shivanna (1971a), however, did not find any beneficial effect of CH for seed development following self placental pollination in *Petunia axillaris*. Balatkova et al. (1977a) studied the effect of IAA, kinetin, tomato juice (TJ), coconut milk (CM) and yeast extract (YE) on seed development following placental pollination in tobacco. Whereas CM, TJ and YE proved inhibitory, the presence of 10 μg l^{-1} IAA or 0.1 μg l^{-1} kinetin significantly improved the number of seeds per ovary. Higher levels of kinetin (1 μg l^{-1}) were inhibitory.

TABLE 10.3

Composition of the modified Nitsch's medium widely used to culture in vitro pollinated ovules[a]

Constituents	Amount (mg l^{-1})
$CaNO_3 \cdot 4H_2O$	500
KNO_3	125
KH_2PO_4	125
$MgSO_4 \cdot 7H_2O$	125
$CuSO_4 \cdot 5H_2O$	0.025
Na_2MoO_4	0.025
$ZnSO_4 \cdot 7H_2O$	0.5
$MnSO_4 \cdot 4H_2O$	3.0
H_3BO_3	0.5
$FeC_6O_5H_7 \cdot 5H_2O$	10.00
Glycine	7.5
Ca-Pantothenate	0.25
Pyridoxine·HCl	0.25
Thiamine·HCl	0.25
Niacin	1.25
Sucrose	50000
Agar	7000

[a]After Kanta and Maheshwari (1963b).

Sladky and Havel (1976) reported the beneficial effect of hen's egg yolk (100 drops l^{-1}) on pollen germination and seed development in the cultures of in vitro pollinated ovaries of maize. However, subsequent workers did not need to add such a complex mixture for seed development in maize (see Table 10.1).

(iii) *CO_2.* In three genotypes of *Trifolium repens* treatment of florets with an elevated level of CO_2 (1%), for 24 h after in vitro pollination, increased the yield of selfed seeds (Douglas and Connolly, 1989).

(iv) *Storage of cultures.* There are hardly any data on the precise effect of light on the response of in vitro pollinated ovules. Cultures are usually stored in darkness or near darkness. Zenkteler (1969) did not find any difference in the results of in vitro pollination whether the cultures were stored in light or dark (see also Zenkteler, 1980).

Balatkova et al. (1977b) have shown that in some systems temperature may influence seed-set. They observed that in *Narcissus* storing the cultures at 15°C, instead of the usual 25°C, resulted in a dramatic increase in the number of seeds developed in each ovary. However, such a low

temperature did not improve seed-set in *Papaver somniferum*, which flowers under comparatively warmer conditions.

Dupuis and Dumas (1990) have reported that in maize the temperature during the first 4 h following in vitro pollination is very critical for fertilization. Incubation of the cultures at 40°C immediately after pollination suppressed fertilization. However, if the elevated temperature was applied 4 h after pollination at normal temperature it did not affect fertilization.

(v) *Genotype*. There is some evidence of genotypic variation in the response of in vitro pollinated ovaries of maize (Gengenbach, 1977b; Bajaj, 1979).

10.4. IN VITRO FERTILIZATION

For years animal scientists have been practising in vitro fertilization to create organisms and to study processes related to gametic fusion and early embryogenesis. This could not be done with higher plants where the egg cell is encased in several layers of ovular tissues. Therefore, the recent success of a German group in fusing isolated male and female gametes of maize (Kranz et al., 1990, 1991) and regenerating, via embryogenesis, fully fertile hybrid plants from the fusion products (Faure et al., 1993; Kranz and Lorz, 1993) is a major breakthrough in the field of plant biotechnology. It opens out the doors to study the molecular events and tissue differentiation during early stages of embryogenesis in flowering plants and offers new opportunities for the genetic engineering of crops.

Since 1987 several papers have described the isolation of male (Dupuis et al., 1987; Cass and Fabri, 1988; Wagner et al., 1989a) and female (Wagner et al., 1989a,b; Kranz et al., 1990) gametes of maize. In 1990 Kranz et al. described their maiden success with electrofusion of an isolated egg with a sperm cell of maize; the in vitro fertilized egg divided and formed mini calli with a high frequency even after being injected with plasmid DNA. More recently, normal hybrid plants have been regenerated from the zygotes formed by in vitro mating of single male and female gametes derived from different inbred lines of maize (Kranz and Lorz, 1993). The techniques of gamete isolation, their in vitro fusion and culture are summarised in the following paragraphs.

The sperms were isolated from pollen grains by osmotic shock in 540 mosmol kg^{-1} H_2O–mannitol solution. Viable egg cells were isolated by microdissection from the ovules incubated, for 40–60 min, at 24 ± 0.5°C, in an enzyme solution containing pectinase (0.75%), pectolyase Y23

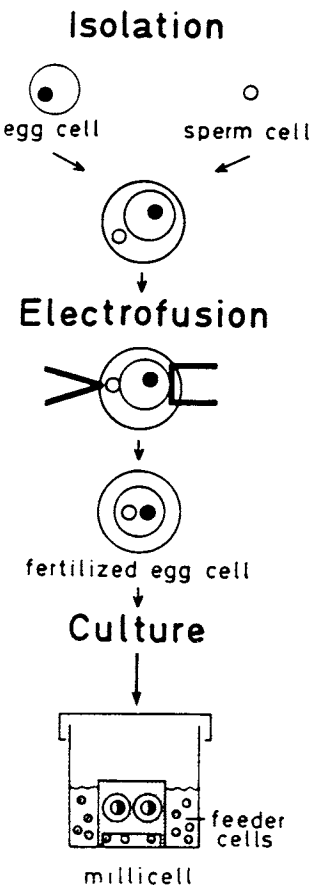

Fig. 10.4. Diagrammatized summary of the method used for electrofusion-mediated in vitro fertilization in *Zea mays*. Single isolated egg and sperm cells were transferred into a fusion droplet and fused electrically after dielectrophoretic alignment on one of the electrodes. For culture the fusion products were transferred, individually, into 'Millicell' inserts surrounded by feeder cells. For details see text (after Kranz et al., 1991).

(0.25%), hemicellulase (0.5%) and cellulase Onozuka RS (0.5%), with osmolarity adjusted to 540 mosmol kg^{-1} H_2O with mannitol, and pH set at 5. With this protocol roughly 5 intact egg cells could be obtained from every 20 ovules tried (Kranz et al., 1991). The freshly isolated gametes are naked protoplasts requiring very delicate handling.

To fuse isolated gametes (Fig. 10.4), single sperm and egg cells were selected and carefully transferred to 1 or 2 μl droplets of fusion solution (540 mosmol kg^{-1} H_2O–mannitol solution) on a coverglass with the aid of microcapillaries connected to a computer-controlled dispenser. The fusion

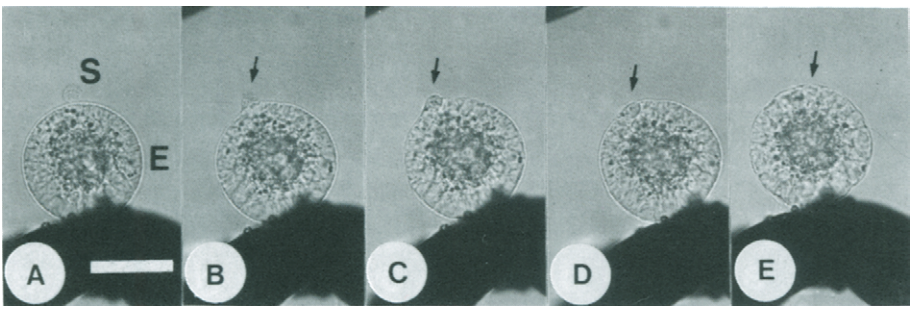

Fig. 10.5. Electrofusion sequence of a selected pair of egg (E) and sperm (S) cells of *Zea mays*. Arrows indicate the fusion site of sperm with egg cell. After the application of DC pulse the time interval from A to E was 4 s (after Kranz et al., 1991).

droplets were covered with a thin layer of mineral oil. Controlled fusion of the gametes was performed with a pair of adjustable electrodes mounted on the microscope. A single DC pulse (0.9–1.0 kV cm^{-1}) for 50 μs, after dielectrophoretic alignment (1 MHz, 71 V cm^{-1}) of the gametes on one of the electrodes for a few seconds, was sufficient to bring about high frequency 'fertilization' (Fig. 10.5). Alignment of the gametes in a manner that the egg was in contact with the electrodes favoured better fusion (Kranz et al., 1991). With this protocol up to 100% fusion could be achieved (mean fusion frequency 79%). Fusion of sperm and egg cells did not occur without an electric pulse even if they were brought into close contact.

The in vitro fertilized eggs were individually cultured on a semipermeable, transparent membrane of a Millicell-CM dish (12 mm diameter) filled with 0.1 ml of nutrient solution. This dish was inserted in the middle of a 3 cm petri plate filled with 1.5 ml of nutrient medium containing feeder cells derived from embryogenic suspension cultures of another maize inbred line. A modified MS medium supplemented with 1 mg l^{-1} 2,4-D and 0.02 mg l^{-1} kinetin, osmolarity adjusted to 600 mosmol kg^{-1} H_2O with glucose and pH set at 5.5 was used. The cultures were maintained under a light/dark cycle of 16/8 h with approximately 50 μE m^{-2} s^{-1} irradiance. Under these conditions karyogamy occurred within 1 h of fusion (Faure et al., 1993) and 92% of the 'zygotes' showed unequal division within 3 days. Under identical conditions unfertilized eggs did not divide. About 90% of the fusion products produced mini colonies and 41% of the cells developed into globular embryos or embryo-like structures (Kranz et al., 1991; Kranz and Lorz, 1993). Full plants could be regenerated by transferring these organized structures, 10–12 days after fusion, to semisolid medium of an altered composition. From 28 fusion products 11

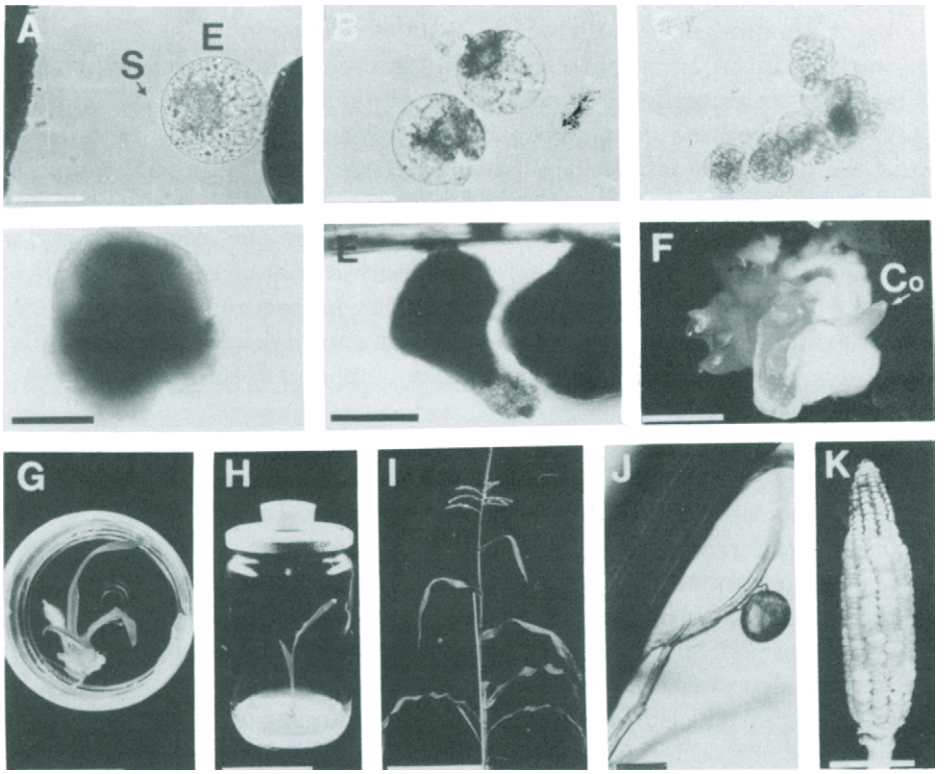

Fig. 10.6. Regeneration of fertile plants from the zygote formed by in vitro fertilization of isolated egg of *Zea mays*. (A) Alignment of an egg cell (E) with a sperm on one of the electrodes before electrofusion. The arrow is pointing at the sperm cell. (B) First polar division of the 'zygote' 42 h after 'in vitro fertilization'. (C) Multicellular structures, 5 days after fusion. (D) Polarized multicellular structure with an outer cell layer at one pole and vacuolized cells at the other end, 12 days after fusion. (E) Transition-phase embryo, 14 days after fusion. (F) A structure with compact white and green tissues, 30 days after fusion; the arrow indicates the coleoptile (Co). (G,H) Plantlets, 35 and 39 days after fusion, respectively. (I) A flowering plant, 99 days after gametic fusion. (J) Germination of a self pollen on the silk of a plant raised from in vitro fertilized egg. (K) A cob, 148 days after in vitro fertilization (after Kranz and Lorz, 1993).

plants were regenerated (a frequency of 48%; Fig. 10.6) within 86 days after fusion (Kranz and Lorz, 1993).

10.5. APPLICATIONS

Several potential applications of in vitro pollination in genetic manipulation of plants have been suggested (Tilton and Russell, 1984; Matthys-Rochon, 1992). The recent success with in vitro fertilization of excised egg of maize has added considerable importance to this area of plant tis-

sue culture. When this new technique becomes applicable to some other species, it should allow study of some novel aspects of sexual reproduction, such as intergametic attraction and gametic recognition, which has not been possible in higher plants so far. Fusion of genetically modified gametes may become another approach to genetic engineering of crop plants. Some of the proven applications of in vitro pollination are discussed here.

In vitro ovular and placental pollinations, where the stigmatic, stylar and ovary wall tissues are almost completely removed from the path of the pollen tube, are potentially very useful in inbreeding and hybridization programmes when the zone of incompatibility lies in the stigma, style or ovary. In vitro pollination has also helped in obtaining haploids.

10.5.1. Inbreeding

Petunia axillaris is a self-incompatible species. In self-pollinated pistils the pollen germination is good, but the pollen tube does not enter the ovary. Rangaswamy and Shivanna (1967, 1971a) have shown that when self-placental pollination is performed in this taxon, fertilization and seed-set occur normally. These authors (Shivanna and Rangaswamy, 1969) could also overcome self-incompatibility in *P. axillaris* through in vivo bud pollination. However, they did not comment on the comparative superiority of one technique over the other. Niimi (1970) reported the development of viable seeds on placenta pollinated by self pollen in another self-incompatible species, *Petunia hybrida*.

Production of homozygous plants of leguminous taxa through androgenesis or gynogenesis has not been successful so far. In the legumes such as *Trifolium* species, with gametophytic incompatibility, even inbreeding to achieve homozygosity is not applicable. Douglas and Connolly (1989) have reported that in *T. repens* the frequency of seed-set following in vitro self-pollination was 5–10 times greater than that in vivo.

10.5.2. Hybridization

Potentially, the most important application of in vitro placental pollination is in raising hybrids which are unknown because of the prefertilization incompatibility barriers. Zenkteler and his associates (see Zenkteler, 1992) attempted several interspecific, intergeneric and interfamily crosses (see Table 10.1) through placental pollination. In the crosses *Melandrium album* × *M. rubrum*, *M. album* × *Viscaria vulgaris*, *M. album* × *Silene schafta* and *Nicotiana alata* × *N. debney* seeds containing viable embryos developed.

Marubashi and Nakajima (1985) produced fertile hybrids with 96 chromosomes by in vitro pollination of *Nicotiana tabacum* with the pollen of *N. rustica*, followed by hybrid ovule culture. This sexually incompatible cross has also been made through protoplast fusion but all the somatic hybrids were aneuploid (Douglas et al., 1981). More recently, DeVerna et al. (1987) raised two interspecific hybrids by in vitro pollination of *N. tabacum* with *N. amplexicaulis*. This cross exhibits unilateral incompatibility. Dhaliwal and King (1978) produced interspecific hybrids by pollinating the exposed ovules of *Zea mays* with pollen grains of *Z. mexicana*.

When ovules of *Nicotiana tabacum* were pollinated with the pollen of *Hyoscyamus niger* the hybrid embryos developed up to the globular stage and well developed endosperm was formed (Zenkteler and Melchers, 1978). The embryo exhibited some budding but did not develop further normally. Excising the ovules and culturing them in a liquid medium containing 2 mg l^{-1} kinetin, 4 mg l^{-1} 2,4-D and 6% sucrose stimulated embryo growth up to the pre-heart shape stage, after which they started to callus. The point of interest in this and several other wider crosses is that one could obtain a cell(s) (zygote/young proembryo) carrying a combination of genomes from desired parents. In such cases, an elaborate attempt to culture the hybrid embryo or to induce organogenesis in the callusing hybrid proembryos would be worthwhile.

10.5.3. Haploid production

Hess and Wagner (1974) raised haploids of *Mimulus luteus* cv. *tigrinus grandiflorus* by pollinating their exposed ovules with the pollen of *Torenia fournieri*. The authors have concluded that the haploids developed parthenogenetically. However, in the absence of detailed anatomical and cytological investigations the alternative origin of the haploids through selective elimination of chromosomes of *Torenia* following fertilization cannot be ruled out (see Section 7.7). Hess and Wagner could not obtain haploids of *M. luteus* through anther culture. Parthenogenetic development of haploid plants in the cultures of unfertilized ovules and ovaries is well known (see Section 7.6).

10.5.4. Production of stress tolerant plants

In vitro pollination at high temperature (38°C) resulted in the production of heat stress-tolerant plants of maize (Petolino et al., 1990). At the elevated temperature only heat stress tolerant pollen grains were able to effect fertilization, and the resulting sporophytes expressed the gameto-

phytic trait. These plants exhibited better agronomic performance (increased seedling vigour, reduced stalk and root lodging, and high grain yield) at high temperatures compared to the plants produced through in vitro pollination at normal temperature (28°C). This approach to gametic selection during in vitro pollination may prove useful to produce plants resistant to other environmental stresses.

10.6. CONCLUDING REMARKS

The technique of in vitro pollination appears very promising to overcome pre-fertilization barriers to compatibility, and raising new genotypes. Although the feasibility of the technique was demonstrated almost three decades ago, for long the plant breeders did not show much interest in applying it to a specific desirable cross. Probably the more fascinating areas of plant tissue culture, such as haploid production through anther and pollen culture and protoplast fusion and culture overshadowed the potential of this simple technique. However, the last decade has witnessed fresh interest in the application of this technique to produce hybrids. Very extensive studies have been made on in vitro pollination and fertilization in maize.

One of the proposed uses of isolated plant protoplasts is in producing hybrids through somatic cell fusion, which involves several tedious steps. One of the most important of these is the selection and identification of the hybrid components from amongst millions of other types of cells, tissues and plants. Although comparative data are not available, it should be easier to identify the hybrid cell in in vitro pollination than in somatic hybridization. In several interspecific and intergeneric combinations in vitro pollination resulted in fertilization and development of young hybrid proembryos but the embryos failed to mature, reflecting our inadequate knowledge of culture requirements of young embryos. In such cases, a detailed search for a suitable nutrient medium to promote the normal development of the hybrid embryo in ovulo or ex ovulo may allow full hybrid plants to be raised. Alternatively, it may be possible to induce organogenesis or embryogenesis in the callusing hybrid embryo; after all, this is the only way of obtaining a hybrid through somatic cell fusion. As a rule, the embryonal callus is more likely to exhibit plant regeneration than the callus derived from somatic cells.

Considerable success has been achieved with isolation of viable male and female gametes of higher plants (Yang and Zhou, 1992). The recent report of plant regeneration from the fusion product of isolated single egg and sperm cells of maize has opened up an entirely new approach to crop improvement (Kranz and Lorz, 1993). Faure et al. (1994) have reported

high frequency (79.7%) fusion of isolated male and female gametes in the presence of 5 mM $CaCl_2$. In this chemical fusion method the gametes preserved the cell recognition system so that the fusion was largely restricted to male–female gamete pairs. They also observed that the egg fertilized in vitro did not fuse with additional sperm, suggesting that a block to polyspermy exists.