Pak. J. Bot., 19(1): 53–59, 1987.

IN VITRO POLLINATION AND DEVELOPMENT OF EXCISED OVULES IN *LYCOPERSICON ESCULENTUM*

M. SARWAR

Plant Genetic Resources Laboratory,
Pakistan Agricultural Research Council, POB 1031, Islamabad, Pakistan.

Abstract

In vitro pollination of *Lycopersicon esculentum* was successful where the placenta with ovules was tranferred onto the cultured pollen. Viability of pollen was less than 5% in pollen germination medium. Use of *in vivo* fertilized ovaries, the age of developing fruit when excised was the limiting factor for its success in culture. Development of ovules was noticed when 10 days old fertilized ovaries were excised and cultured on Linsmaier & Skoog medium; which was found better than other media.

Introduction

Formation of viable seeds *in vitro* after the placement of pollen to the surface of excised placenta containing ovules was first described by Kanta *et al.*, (1962). The placental pollination of ovules can overcome self-incompatibility when the barrier lies in stigma and style. Basic technique of placental pollination has proved successful in some cases and self-incompatibility was overcome in *Petunia exillaris* (Rangaswamy, 1977), and in interspecific hybridization (Slusarkiewicz-Jarzina, 1984; Fassuliotis & Nelson, 1985). Ovules in which fertilization had occurred enlarged rapidly and underwent normal development into seeds containing viable embryos (Rangaswamy & Shivanna, 1967). In most cases the success in the growth of ovules is related to the age (Rangan *et al.*, 1969) and size (Kunert & Peterka, 1984) of embryos at the time of culture.

For successful embryo culture, it was attempted to follow (1) comparison of different media, (2) make observation of the relationship between ovary size at the time of culture and its growth in culture, and (3) study the development of a technique for *in vitro* pollination in tomato which would assist the future breeding programmes for interspecific and intergeneric hybridization.

Meterials and Methods

Plant material: Seeds of tomato (*Lycopersicon esculentum* var. Moneymaker) were sown and plants raised under glasshouse. When petal started to turn yellow the flower buds were emasculated for *in vitro* and *in vivo* pollination.

Pollen viability and germination: Pollen viability was tested with acetocarmine and fluorescein techniques (Heslop-Harrison & Heslop-Harrison, 1970). Freshly collected pollens were sprinkled on 4 media under study and their germination tested at 20°C, and in pollen germination medium (Belatkova & Tupy, 1968). For *in vitro* pollination, pollens were collected under aseptic conditions as described by (Petru *et al.*, (1964). Fertilized and unfertilized ovaries excised from the plant were surface sterlized for 10 min. in 10% solution of sodium hypochlorite.

In vivo and in vitro pollination:

(a) *In vivo* fertilized ovaries were excised at 2 day interval upto 10 days after pollination. They were grouped into 3 categories according to their size as measured by Vernier caliper (mm); 0.34-0.37, 0.42-0.45, 0.70-0.73. Unfertilized ovaries were cultured as control.
(b) For *in vitro* pollination 3 methods employed were:
   i. Stigmatic pollination – Pollens were transferred directly to the stigma, and ovary with stigma was cultured.
   ii. Transfer of pollens onto the cultured placenta with ovules – ovaries were cut into halves and each half with cut end up was placed on the media. One drop of cultured pollen in a germination medium was poured onto each half.
   iii. Transfer of placenta with ovules onto cultured pollen – a drop of cultured pollen germination medium was poured onto the media. Ovaries were cut into halves and each half with cut surface down was placed onto the cultured pollen.

In vitro pollination and growth of ovules was determined by the change in colour of the ovules.

Media: The agar solidified media having 2% sucrose were of 4 types with the following ingredients in concentrations of mg/l as indicated.

i) Linsmaier & Skoog (1965) medium (LS) + 2, 4-D (15) + L-lysine (183) + L-glutamic acid (147) + L-serine (205) + 0.7% agar.
ii) Murashige & Skoog (1962) medium (MS) + IAA (0.3) + kinetin (0.04-10) + 0.4% agar.
iii) Nitsch & Nitsch (1969) medium (N) + 0.4% agar.
iv) White (1954) medium (W) + 0.4% agar.

The pH of media was adjusted to 5.6 before mixing agar, autoclaved and poured into 5 cm plastic Petri dishes. Three replications of each treatment were incubated under 16 h/day of 200 lux at 25° ± 1°C.

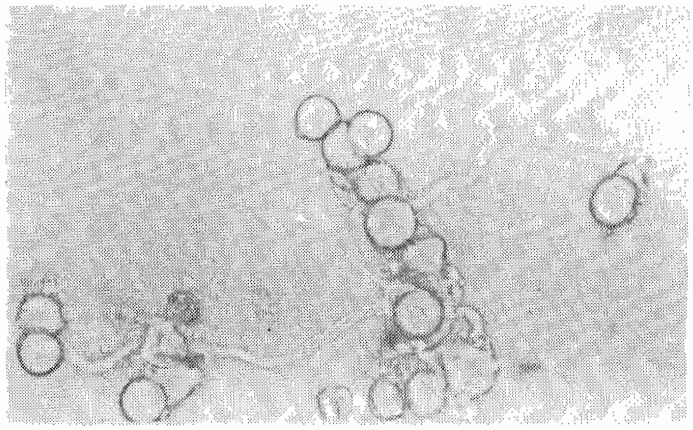

Fig. 1. Germination of pollen grains in the pollen germination medium with fluorescein, showing different stages of pollen tube growth. (x 420).

Procedure for embedding, sectioning and staining was used as described by Johansen (1940). Scanning electron microscope studies were conducted as done by Vasil & Vasil (1984).

Results

Freshly collected pollens did not germinate on any of the 4 media at 20°C; with acetocarmine the viability was 90%. Pollens showed less than 5% viability in pollen germination medium with fluorescein technique (Fig. 1). Pollens, however, produced normal commercially acceptable level of fruit on greenhouse raised plants.

In vivo pollination

In culture, pollinated and unpollinated ovaries responded differently. The former grew to form miniature seed while the later failed to gain size. The *in vivo* pollinated ovaries grew for first fews days on all the media. Ovaries excised 2, 4, and 8 days after pollination took 20, 16 and 10 days, respectively, to change to red colour. The developing ovules showed a marked shrinkage and depression in the centre compared with the normal seed (Fig. 2). When the ovaries were excised 10 days after pollination, their size in 0.70-0.73 mm range, changed to red colour after the fourth day of culture (Fig. 3a). Ovules in culture showed normal development initially on LS medium but the breakdown of their placental and embryonic tissue started and increased with time (Fig. 4). On cross section of the cultured ovaries, degeneration of placental tissue was noted in the first week of culture. Ten days after culture an advanced stage of ovule shrinkage and degeneration of placental tissue was noted.

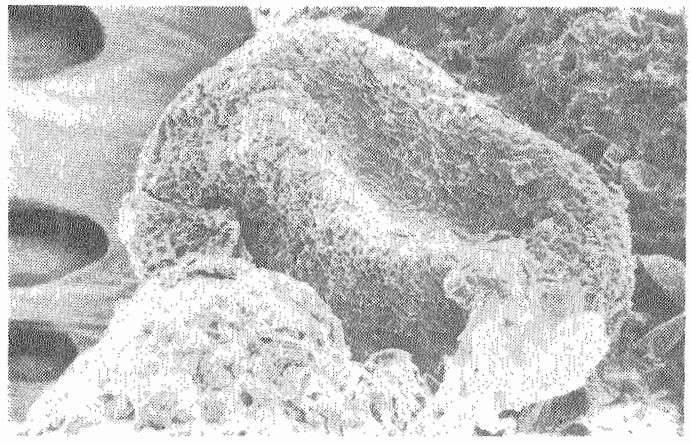

Fig. 2. Electorn micrograph of *in vitro* developing ovule taken out of culture after 10 days, showing depression and shrinkage of developing ovule. (x 420).

In vitro pollination

i. *Stigmatic pollination:* Ovaries started to swell and increased in size as compared to unfertilized ones.
ii. *Transfer of pollens on to the cultured placentae with ovules:* No swelling of the ovules or the ripening of the placental tissues was noted.
iii. *Transfer of placenta with ovules onto cultured pollen:* After 20 days of pollination, swelling of the ovules was observed. The enlargement of 25-35 naked ovules showed that the pollen tubes have entered the embryo and fertilization has occurred (Fig. 3b). The ovaries retained the original (*in vivo*) arrangement of ovules on the placenta. Swelling of ovules occurred on LS medium only.

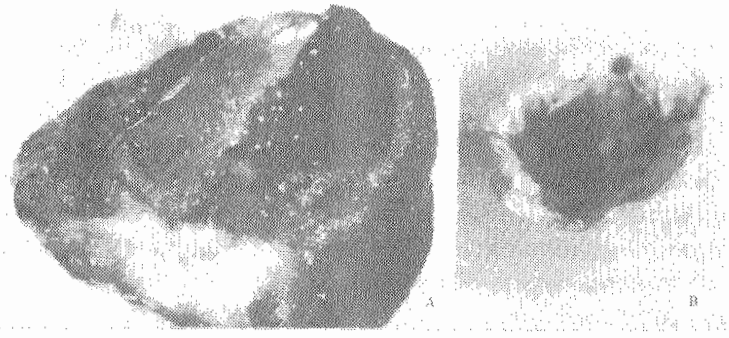

Fig. 3. a – Light micrograph of developing ovules attached with the placenta. *In vitro* fertilized ovary was excised 10 days after pollination. b – Light micrograph showing developing ovules 20 days after *in vitro* fertilization. (x 420).

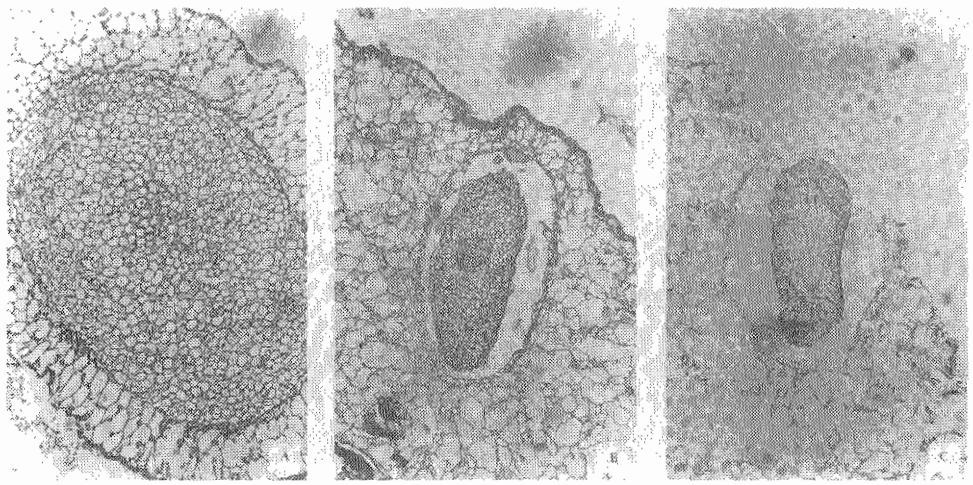

Fig. 4. Cross-section of light micrograph of developing ovules attached with placenta showing different stages of braekdown of placental and embryonic tissues. a – Normal development of ovule and placental tissues after 4th day in culture. b – Breakdown of ovule and placental tissues within 6 days after culture. c – Shrinkage of ovule and disintegration of placental tissue, 10 days after culture. (x 560).

Discussion

In the present investigations, attempts were made to devise a technique for cultured ovary to remain alive and grew in isolation from the mother plant. The degeneration of the endosperm and shrinkage of the ovules indicated the lack of nutrient and other exogenic factors. This could be due to the osmotic concentration of the nutrient medium. The sucrose concentration of 2% was used as a carbon source which seems to fulfill the osmotic requirements of 10 days old ovules while the requirements of young ovules seems different which could be higher. This supports the earlier work reported by Wakizuka & Nakajima (1974). Usually embryo begin to grow exponentially when it consists of 10-20 cells, this cell number is related to the age and size of the embryo at the time of culture. Ten-day-old *in vivo* fertilized ovaries were in 0.70-0.73 mm range, the size was large as compared to the ovaries excised 2 to 8 days after pollination. This shows that the embryo size, which is related to the age, is an important aspect in the successful development of ovules in culture. Rangan *et al.*, (1969) successfully cultured 100-200 days old fertilized ovaries of citrus. Later, Kunert & Peterka (1984) found a positive correlation between embryo size and plant forming embroys.

Information is not available on the effects of agar, temperature, and light on the embryo development in culture. The high agar concentration could have exerted positive effect on embryo development. The LS medium contained 0.7% agar while other media

had 0.4% agar. Swelling of ovaries occured on LS medium only. This medium contained 2, 4-D and othe organic additives which were not present in other media confirming the superiority of 2, 4-D over IAA in the success of embryo culture.

In placental pollination the ovaries were cut into halves to produce 25-35 naked ovules with placenta. *In vitro* pollination technique, where the drop of pollen medium was poured onto the cultured ovules, did not lead to fertilization which might be due to the presence of water in the pollen medium as water is known to inhibit the growth of pollen tube (Zenkteller, 1984). In the other two techniques, water was excluded from the pollination and fertilization processes. Water in the pollen medium was allowed to evaporate before the placentae with ovules were cultured (Fig. 3). The pathway of pollen tube growth through the stigma, style, and placenta to the embryo sac is a natural process which leads to fertilization and formation of viable seed. The placental pollination is superior to stigmatic ones when the natural barrier of incompatibility zone exists in stigma and style and offers opportunity to produce interspecific and intergeneric hybrids which occur rarely in nature.

References

Balatkova, V. and J. Tupy. 1968. Test tube fertilization in *Nicotiana tabacum* by means of an artificial pollen tube culture. *Biologia Plant.*, 10: 266-270.

Fassuliotis, G. and B.V. Nelson. 1985. Plant regeneration from embroys of *Cucumis metuliferus* cross-pollinated with *Cucumis anaguria.* In: *Tissue Culture in Forestry and Agriculture.* (Eds.) R.R. Henke, K.W. Hughes, M.J. Constantin and A. Hollaender. Plenum Press, New York, USA. p. 315.

Heslop-Harrison, J. and Y. Heslop-Harrison. 1970. Evaluation of pollen viability by enzymatically induced fluorescence: intracellular hydrolysis of fluorescein diacetate. *Stain Tech.*, 45: 115-120.

Johansen, O.A. 1940. *Plant Microtechnique.* McGraw-Hill Book Co., New York, USA.

Kanta, K., N.S. Rangaswamy and P. Maheshwari. 1962. Test tube fertilization in a flowering plant. *Nature,* 194: 1214-1217.

Kunert, R. and H. Peterka. 1984. Effectiveness of embryo culture in haploid barley production in relation to embryo differentiation and size. In: *Plant Tissue and Cell Culture Application to Crop Improvement* (Eds.) F.J. Novak, L. Havel and J. Dolezel. Czechoslovak Academy of Sciences, Prague. pp. 255-256.

Linsmaier, E.M. and F. Skoog. 1965. Organic growth factor requiremnts of tobacco tissue culture. *Physiol. Plant.*, 18: 100-127.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.*, 15: 473-497.

Nitsch, J.P. and C. Nitsch. 1969. Haploid plants from pollen grains. *Science,* 163: 85-87.

Petru, E., E. Hrabetora and J. Tupy. 1964. The techniques of obtaining germinating pollen without microbial contamination. *Biologia Plant.*, 6: 68-69.

Rangan, T.S., T. Murashige and W.P. Bitter. 1969. *In vitro* studies of zygotic nuclear embryogenesis in citrus. *Proc. 1st Citrus Symp.*, 1: 225-229.

Rangaswamy, N.S. 1977. Application of *in vitro* pollination and *in vitro* fertilization. In: *Plant Cell, Tissue and Organ Culture* (Eds.) J. Reinert and Y.P.S. Bajaj. Springer-Verlag, Berlin, W. Germany. pp. 412-425.

Rangaswamy, N.S. and K.R. Shivanna. 1967. Induction of gamete compatibility and seed formation in axenic cultures of a diploid self-incompatible species of Petunia. *Nature*, 216: 937-939.

Slusarkiewicz-Jarzina, A. 1984. The test tube pollination of ovules in some species of *Solanaceae*. In: *Plant Tissue and Cell Culture Application to Crop Improvement* (Eds.) F.J. Novak, L. Havel and J. Dolezel. Czechoslovak Academy of Sciences, Prague. pp. 587-588.

Vasil, V. and I.K. Vasil. 1984. Preparation of cultured tissues for scanning electron microscopy. In: *Cell Culture and Somatic Cell Genetics of Plants. Vol. I. Laboratory Procedures and their Applications* (Ed.) I.K. Vasil. Academic Press, New York, USA. pp. 738-743.

Wakizuka, T. and T. Nakajima. 1974. Effect of cultural condition on the *in vitro* development of ovules of *Petunia hybrida. Japan J. Breed.*, 24: 182-187.

White, P.R. 1954. *The cultivation of animal and plant cells.* The Ronald Press, USA.

Zenkteller, M. 1984. *In vitro* pollination and fertilization. In: *Cell Culture and Somatic Cell Genetics of Plants. Vol. I. Laboratory Procedures and their Applications* (Ed.) I.K. Vasil. Academic Press, New York, USA. pp. 269-275.

(Received for publication 14 May, 1986)