Ovary and embryo culture

Culture of unfertilized ovaries to obtain haploid plants from egg cell or other haploid cells of the embryo sac is called ovary culture and this process is termed as gynogenesis. San Noem first reported the gynogenesis in barley in 1976. Subsequently, success has been obtained in several species including wheat, rice, maize, tobacco, sugar beet, rubber etc. About 0.2-6% of the cultured ovaries show gynogenesis and one or two, rarely up to 8, plantlets originate from each ovary. The rate of success varies considerably with:

- ✓ Species
- ✓ Markedly influenced by the genotype so that some cultivars do not respond at all. e.g. In rice, *japonica* genotypes are far more responsive than *indica* genotypes.
- ✓ Stage of ovary development. In most cases, the optimum stage for ovary culture is the nearly mature embryo sac, but in rice ovaries at free nuclear embryo sac stage are the most responsive. Generally, culture of whole flowers, ovary and ovules attached to placenta respond better, but in *Gerbera* and Sunflower isolated ovules show better response. Cold pretreatment (24-48 hr at 4°C in sunflower and 24 hr at 7°C in rice) of the inflorescence before ovary culture enhances gynogenesis.
- ✓ Growth regulators:

  Growth regulators are crucial in gynogenesis and at higher levels they may induce callusing of somatic tissues and even suppress gynogenesis. Growth regulator requirement seems to depend on species. For example, in sunflower, GR-free medium is the best, while even at low level MCPA (2-methyl-4-chlorophenoxyacetic acid) induces somatic calli and SEs. But in rice, 0.125-0.5 mg/l MCPA is optimum for gynogenesis.
- ✓ Other Factors:

  Sucrose level also appears to be critical. In sunflower, 12% sucrose leads to gynogenic embryo production, while at lower levels somatic calli and somatic embryos were also produced. Ovaries/ ovules are generally cultured in light, but atleast in some species, e.g., sunflower and rice, dark incubation favours gynogenesis and minimises somatic callusing. In rice, light may lead to degeneration of gynogenic proembryos.

Developmental stages

Generally, gynogenesis has two or many stages and each stage has distinct requirements. In rice, tow stages *viz*., induction and regeneration are recognized. During

induction, ovaries are floated on a liquid medium having low auxin and kept in dark, while for regeneration they are transferred on to an agar medium with higher auxin concentration and incubated in light.

Haploid plants generally originate from egg cell in most of the species (in vitro parthenogenesis) but in some species, e.g., rice, they arise chiefly from synergids; in atleast *Allium tuberosum* even antipodals produce haploid plants (in vitro apogamy). As in anther culture, gynogenesis may occur either via embryogenesis or through plantlet regeneration from callus. In rice MCPA generally leads to a small amount of protocorm like callus formation from which shoots and roots regenerate, while picloram promotes embryo regeneration. In contrast, sugarbeet usually shows embryo development, while in sunflower embryos regenerate following a callus phase. In general, regeneration from a callus phase appears, at least for the present, to be easier than direct embryogenesis.

Advantages

1. Gynogenetic haploids may be a valuable substitute for the production of homozygous lines in cases where cytoplasmic male sterility prevents the use of micropsores.
2. Reduction in the frequency of albino plants in some species especially cereals.

Limitations

1. So far it has been successful only in less than two dozens species.
2. The frequency of responding ovaries (1-5%) and the number of plantlets/ovary (1-2) is quite low.
3. Therefore, anther culture is preferred over ovary culture. Only in those cases where anther culture fails, e.g. sugarbeet and for male sterile lines, ovary culture assumes significance.

Embryo culture

In angiosperms the embryo is the miniature sporophyte resulting from the fertilized egg or zygote. In seed bearing plants, embryos are easily accessible as they can be separated with relative ease from the maternal tissues and cultures *in vitro* under aseptic conditions in media of known chemical composition. The culture of embryo has been practiced by plant breeders for over half a century.

The first systematic attempt to grow the embryos of angiosperms *in vitro*, under aseptic conditions was made by Hanning (1904) who cultured mature embryos of *Raphanus* and the conifers *Cochlearia*. Subsequently, many workers raised plants by cutting embryos excised from mature seeds. Further progress in the field of embryo culture was provided by Liabach (1925, 1929) who demonstrated the most important practical application of this technique. He crossed *Linum perenne* with *Linum austriacum* but obtained hybrid seeds of very light and shriveled nature without any germinability. The excised embryos from such seeds were cultured on moist filter paper dipped in sucrose solution. This led to the production of hybrid plants. Since then, the technique of embryo culture has been widely used to produce hybrids which were otherwise not possible due to embryo abortion. Further, embryo culture method offers new refined ways to characterise the development of embryo and related problems in plants.

The selection of plant to be used for embryo culture is normally dictated by the problem in hand. When the goal is to obtain plants from otherwise abortive seeds, the embryos should be excised for culture prior to the onset of abortion. Zygotic embryos, being enclosed within the sterile environment of the ovular and ovarian tissues, do not require surface sterilization. Entire ovules are disinfected following the standard methods of surface sterilization and embryos are dissected out and transferred to culture medium under aseptic conditions.

For the *in vitro* culture of embryo generally, it is necessary to excise them from their surrounding tissues. The mature embryos can be isolated with relative ease by splitting open the seeds. Seeds with a hard seed coat are dissected after soaking them in water. For plants with minute seeds, the isolation of embryos can be done under dissecting microscope on a sterilized slide. In plants like, orchids, where the seeds are minute and lack functional endosperm the entire ovules having embryos are cultured on the medium.

Types

- Culture of immature embryos originating from unripe seeds that is mainly to avoid embryo abortion with the purpose to produce a viable plant.
- Culture of mature embryos derived from ripe seeds.

Factors affecting the success of embryo culture

Before attempting to elucidate the application of embryo culture method, it is necessary to analyze briefly the factors influencing the embryo culture technique.

- ✓ Genotypes
- ✓ Developmental stage of the embryo at isolation. The culture of very young embryos is very difficult. Despite considerable progress in the field of embryo culture, embryo rescuing seems to be difficult where embryo abortion occurs at a very early stage of development. To culture very young embryos successfully, the embryo of a particular species is implanted in the endosperm from another seed of the same species. For example, in the cross of *Hordeum x Secale* the survival rate with the implantation technique was 30-40 per cent as compared to one per cent with traditional method of embryo culture. This technique is termed as embryo-nurse endosperm transplant technique.
- ✓ Growth conditions of the mother plant
- ✓ Composition of the nutrient media

  The most important aspect of the embryo culture is the selection of the right culture medium that would support progressive and orderly development of embryos excised at different stages of development. The requirement of culture medium depends on the types of embryo culture. They may be either post-germinal or pre-germinal. In the case of post-germinal embryo culture, embryos are cultured only to speed up the process after germination. This can be achieved with less complex medium or even with sucrose or glucose solution. In pre-germinal embryo culture, immature embryos are cultured to get plantlets, where the embryos require a complex nutrient medium. Refinement of nutrient medium for the culture of embryos includes modifications in the composition of mineral salts, organic nutrients and growth regulators, as for any other type of plant tissue cultures.

  The composition of the culture medium has to be formulated in such a way to suit the developmental phase of the embryo. There are two phases in embryo development (1) heterotrophic phase in which the embryo draws its nutrients from the endosperm and the surrounding maternal tissues and (2) autotrophic phase in which the embryo is metabolically capable of synthesizing substances required for growth.

  Addition of amino acids and vitamins, promoted the development of the embryo. Casein hydrolysate, an amino acid complex has been widely used as an additive to the embryo culture media. The natural plant extracts like coconut milk, tomato juice and extracts of banana produce higher recovery of growth and development of embryos.

Growth hormones, especially auxins are not used in embryo culture media because of their inhibitory role in embryo growth resulting in structural abnormalities.

- ✓ ***Suspensor and embryo culture***

  The suspensor is transitory structure found at the radicle end of the proembryo. It promotes the growth of the young embryos and degenerate in the later stages of embryo growth, i.e., after the formation of cotyledons. Mostly embryos cultured without the suspension showed lesser survival and maximum necrosis thus reducing the frequency of plantlet formation.
- ✓ Light
- ✓ Temperature

Practical applications

Embryo rescue in wide crosses

In plants the embryo inviability occurs due to many causes, though there is normal fertilization and development in the early stage. The impairments start subsequently, resulting in the eventual death of embryo or from the endosperm or from the surrounding maternal tissue.

To overcome the above barriers for obtaining the hybrids, the embryo culture technique is effective utilized in which the nutritional relationship between the embryo and endosperm is restored by providing the artificial medium to induce and complete growth of hibrids embryos and is called as embryo rescuing. The demonstration of the ability of the excised embryos from non-vialbe seeds to grow successfully in artificial medium supplied with nutrients bypassed the problems of wide hybridization and to enable transferance of resistant genes for pests and diseases and various environmental stresses into the cultivated species.

The embryo culture technique is not only adopted to produce interspecific hybrids, but aslo extended to produce viable hybrids between genera. Intergeneric hybrids have been obtained between *Hordeum* and *Secale*; *Hordeum* and *Hordelymus, Triticum* and *Elymus; Triticum* and *Secale* and *Tripsacum* and *Zea*.

However, for the successful embryo rescuing in interspecific and intergeneric crosses, the composition of the artificial nutrient medium is very important. The reason is that the medium formulated to foster growth of embryos of one hybrids combination may not

be suitable for another. To overcome the constraints in the artificial medium in inducing the growth of embryos, the following technique is followed in which the hybrid embryos embedded in hybrid endosperm are removed and transplanted or implanted into the normal endosperm. This technique is termed as embryo implantation. This tecnique was first proposed by Pissarev and Vinogradova in 1944. The embryo implantation technique could be an alternative to improve the crossability between two species. Kruse (1974) proposed a similar method to rescue hybrid embryos from *Hordeum* x *Triticale*, *Hordeum* x *Agropyron* and *Hordeum* x *Secale* crosses. The hybrid embryo is removed from a dehulled caryopsis and placed in the correct position in the endosperm of *Hordeum* placed in a culture medium.

Monoploid production

The advantages of haploids as tools in genetics or plant breeding become more apparent because of their following utility values

- they provide the quickest possible way to get homozygosity
- they may serve to recover recessives
- the gametes of monoploids remain as best source for linkage studies
- the doubled products of monoploids from crosses provide stable recombinants
- the monoploids are useful in genome homology studies
- the monoploids are ideal objects for mutation studies
- the monoploids are useful in gene transfer studies

Considering the above mentioned advantages, monoploid induction and regeneration is considered as a powerful tool in plant breeding. The details of monoploid production from microspores have been described in the chapter on Anther Culture. Here how the embryo culture technique could be exploited for monoploid production is discussed. The technique, popularly known as Bulbosum technique is exploited for producing the monoploids and is based on making an interspecific cross with *Hordeum vulgare* as the female and *H. bulbosum* as male. In this cross fertilization of *H. vulgare* by *H. bulbosum* proceeds normally. During zygote development, the chromosomes of *H. bulbosum* are eliminated from the cells of the developing embryo. The endosperm starts developing and then degenerates. At this stage, the embryonic cells harbour only the set of *H. vulgare* genome and show poor rate of division resulting in smaller haploid embryos. These smaller haploid embryos with little endosperm are dissected out and cultured *in vitro* to produce the haploids. Following *in vitro* embryo culture, the developing haploid plantlets of *Hordeum vulgare* are reared and raised under normal green house conditions and chromosome

doubling is induced on established plants. This method has the advatage of throwing very high frequencies of monoploid (haploid) induction.

Overcoming seed dormancy

The other major application of embryo culture in breeding is as a means of overcoming seed dormancy. Seeds of certain species germinate very slowly or not at all under normal conditions. The cause may be in the form of endogenous inhibitors, lesser length, high temperature, storage condition and maturity of the embryo. These problems can overcome by providing specific signals for seed germination, rightly through embryo culture. Examples include *Iris, Ilex, Viburnum, Paeonia, Brassica chinensis, Musa bulbisiana, etc*.

Shortening breeding cycle of plants

Embryo culture is also useful in reducing the breeding cycle of new varieties in cases where long dormancy causes extension of breeding cycle. Cultivated varieties of rose generally take about a year to flower and two to three months for the formation of fruits. Seedlings produced from cultured embryos flower in two to three months. These flowers can serve as the male parent for further crosses, thus enabling the breeder to produce two generations in one year or shortening the breeding cycle to three or four months. Other example is weeping crap apple (Malusop) in which the seeds cultured *in vitro* produce seedling in four months. On the other hand, seeds planted in the soil take about nine months to germinate.

Combining embryo culture and back crossing in gene transfer

The embryo culture has been proved as a viable technique for resynthesising some of the plant hybrids .For example *Brassica napus* has been resynthesised from the cross of *B. campestris/B. oleracea* using embryo culture. The recent approach is back crossing the resynthesised *B. napus* (2n=38) to *B. campestris* (2n=20), so that the genes from *B. oleracea* can be transferred to B.campestris .In 1988 **Quazi** made an attempt in this regard and came out with sucessful results. He got aline from the back crosses of (*B. napus/B. oleracea*)/*B. oleracea* which is resistant to cabbage aphid attack. Following the same approach **Milanova** and his co-workers (1991) produced cytoplasmically male sterile tobacco plants from *Nicotiana africana* and *N*. *tabaccum* cross. Thus the scheme facilitates gene transfer overcoming the species barrier.

Other applications

The embryo culture technique can be effectively engaged in seed testing of various tree species, germinating seeds of obligate phanerogamic parasites, studying the host-pathogen relationship in seed-borne diseases and studying developmental embryogenesis. The embryo culture technique has already established its creditability as an invaluable tool in plant breeding and advances in embryo culture method have served to open new vistas in the field of *in vitro* culture. But greater attention has to be paid to solve the minute intricacies which remain as big hurdles in the exploitation of embryo culture.

Embryo rescue

Distant crosses may fail due to one or more of several reasons such as inability of pollen to germinate, failure of pollen tubes to grow or perhaps more commonly degeneration of endosperm. When embryo fails to develop due to endosperm degeneration, embryo culture is used to recover hybrid plants. This is called as hybrid rescue through embryo culture. Some recent examples are the recovery of hybrids from *Hordium vulgare X Secale cereale, Triticum aestivum X Agropyron repens, H. vulgare X Triticum aestivum etc.,* In case of *Triticale* rare combinations between *Triticale and Secale* develop viable seeds. But most of the tetraploid and hexaploid wheat carry two dominant genes, *Kr1* and *Kr2,* which prevent seed development in crosses with *Secale*. The majority of the hybrid seeds is small, poorly developed and show very poor germination. Further, seeds are obtained from only 5-10% of the florets pollinated. The recovery of hybrid seedlings is much greater (50-70%) when embryos from 10-14 day old caryopses are removed and cultured on a suitable medium.

Bulbosum technique

Principle

The fertilization proceeds readily between *H. vulgare* and *H. bulbosum.* Zygote induction is high and chromosomes of *H. bulbosum* are rapidly eliminated from the cells of developing embryo. This develops for two to five days and then aborts. In the developing monoploid embryo cells, the division and increment is slower than the diploid cells. This comparatively slow growth of the monoploid condition, together with the disintegration of the endosperm leads to the formation of small embryos which have to be dissected out of the fruits and provided with nutrients *in vitro* in order to complete their development. Following *in vitro* embryo culture, the developing plantlets are raised under normal green house conditions and chromosome doubling is induced on established plants.

Advantages

- The method of hybridization followed by chromosome elimination proves to be of general interest for haploid production in other species of *Hordeum* and also of hexaploid wheat.
- It is possible to produce monoploids of barely in a cytoplasm of *H. bulbosum* by using *H. vulgare as* male and *H. bulbosum* as female. Using embryo culture as vehicle, high frequency foreign cytoplasm monoploids can be obtained.
- *Hordeum* species is not the only one where chromosome elimination is found in higher plants. In Haplopoppus, monoploids have been examined with only two chromosomes. *H. bulbosum* need not be the ideal partner for *H. vulgare* to induce monoploids of barley via somatic chromosome elimination. There can be a range of *Hordeum* that might be tried as a more efficient pattern than *H. bulbosum*.

Questions

1. Embryo culture is used ……

a) To overcome embryo abortion b) To overcome seed dormancy

c) Embryo rescue in distant hybridization **d) All the above**

2. Ovary culture is first reported by ……

a). Northern **b). San Noem** c). Kano d). None of the above

3. Ovary culture is first reported in ……

a). Barley b). Sorghum c). Cotton d). None of the above

4. Embryo culture is first reported in ……

a). Raphanus b). Sorghum c). Cotton d). None of the above

5. Embryo culture is first reported by ……

a). Hanning b). San Noem c). Kano d). None of the above

6. The success of embryo culture depends on ……

a). Developmental stage of the embryo at isolation b). Growth conditions of the mother plant

c). Composition of the nutrient medium **d). All the above**

7. The growth hormone not used in embryo culture medium is ……

a). Auxin b). Gibberellin c). Cytokinin d). None of the above

8. The embryo imlantation technique was proposed by ……

a). Pissarev b). Vinogradova **c). Both a & b** d). Kruse

9. The embryo culture is used for ……

a). Embryo rescue b). Monoploid production

c). To overcome seed dormancy **d). All the above**

10. Bulbosum technique is used for ……

a). Embryo rescue b). Monoploid production

c). To overcome seed dormancy d). All the above

Additional readings…

http://www.youtube.com/watch?v=m5JEZq0Fxuk&feature=related - video