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IN VITRO ZYGOTIC EMBRYO CULTURE

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1. INTRODUCTION

The egg after fertilization forms Zygote which, in turn, passes through a series of well-defined developmental stages to form an embryo, the precursor of the next generation. The embryo growth and development is determined by many physical and chemical factors. The neighbouring tissues, particularly the endosperm, also regulate the programmed pattern of embryo development. Any disruption in these elements leads to aberrations and, in ultimate cases, abortion of the embryo. Valuable information on the developmental and physiological facets of embryogenesis has been offered by *in vitro* embryo culture. It is one of the tissue culture techniques which unearthed applications before it got optimized. It is now extensively employed to produce rare hybrids which otherwise fail due to sexual incompatibility. This chapter explains the method of embryo culture, its significance in understanding the physiology and developmental biology of embryo development and, finally, its applications.

2. HISTORY

Hannig (1904) made the first organised effort to culture embryos of flowering plants *in vitro*. He cultured isolated mature embryos of various Brassicaceae members on a mineral salt medium along with sucrose and got seedlings which could be transplanted. Dieterich (1924) cultured mature embryos of various species on a semi-solid medium with minerals and 2.5-5% sucrose and reported its normal growth. However, the embryos taken from undeveloped seeds did not attain maturity, and instead, germinated directly, bypassing the transitional embryogenic stages.

Laibach (1925, 1929) made interspecific crosses between *Linum perenne* and *L. austriacum* and observed significantly shrunken light seeds that could not germinate. He attempted to germinate embryos removed from these seeds before abortion on a cotton wad or filter paper dipped in sucrose or glucose solution and could grow full hybrid plants. This procedure has since been commonly applied to develop various hybrids which did not succeed in nature due to post-zygotic sexual incompatibility barriers. This method of developing embryos independent of the ovule milieu offers tremendous prospects to investigate the physical and chemical factors affecting embryogenic development. Raghavan (2003) and Thorpe and Yeung (2011) have dealt with the subject in an outstanding manner.

3. TECHNIQUE

The embryo culture technique has two key facets: (i) culture medium composition, and (ii) excision of embryo from the ovule. The composition of the culture medium differs with the material and the age of the embryo to be cultured. Culture of isolated zygotes and very young proembryos is a challenging process, as the media formulations developed have given limited success till date. These explants need co-cultivation with feeder cells/nurse tissue, like embryogenic microspores or robustly developing suspension cell congregates.

To dissect an immature embryo, an ovule, placed in a drop of culture medium on a glass slide or petri plate is split along its length using a sharp mounted blade. The whole embryo along with the suspensor is separated by cautiously teasing apart the ovular tissues. In order to dissect and culture older embryos, a small slit is created using a blunt needle in the ovule on the side which does not have the embryo, and the embryo is pushed out by applying slight pressure.

The spikelets of cereals, are detached from the plant at a suitable stage, surface sterilized and washed carefully before dissecting out the zygote in an appropriate medium.

For culturing zygotes of rice, Zhang *et al*. (1999) employed Kao and Michayluk (1975) medium supplemented with 9% maltose.

4. CULTURE REQUIREMENTS

4.1. Mineral salts

Brown (1906) applied embryo culture technique to study the relative efficacy of different organic nitrogenous compounds on the growth of excised barley embryos and demonstrated that the amino acids, aspartate and glutamate, and the amide, asparagine are better nitrogen sources, causing increased dry weight and nitrogen content of the cultured embryos.

Various mineral salts have been used for embryo culture without analysing the function of specific salts. When Monnier (1976) investigated the influence of various mineral salts in zygotic embryo culture of *Capsella bursa-pastoris*, he concluded that no relationship could be found between the *in vitro* growth and survival of embryos. Maximum growth was reported on MS medium but the survival frequency of the embryos was very low. Survival was the best on Knop's medium but embryos did not grow much. After a comprehensive analysis, Monnier developed a medium which could support good growth of the embryo similar to MS medium as well as high survival. As compared to the MS medium, this medium contained higher K^+ and Ca^{2+} and low NH_4^+concentrations.

For immature embryos of many plants, such as barley (Umbeck and Norstog, 1979), NH_4^+ was either essential or a preferred source of inorganic nitrogen due to the lack of necessary enzyme to reduce NO_3^- to NH_4^+ at the early stage of embryogenesis.

4.2. Amino Acids and Vitamins

Asparagine was found to be very effectual in improving the growth of embryos (Hannig1904). Glutamine has been recognised to be the most efficient amino acid for *in vitro* growth of embryo explants (Matsubara, 1964). Casein hydrolysate, which is a complex mixture of amino acids, has been extensively employed as an additive to culture media, especially for culturing young embryos (Rangaswamy, 1961).

Vitamins have not been found to be mandatory for embryo culture though they have been commonly used in the culture media.

4.3. Carbohydrates

Sucrose is by far the preferred carbohydrate most commonly used for embryo culture. Sucrose when added to the medium not only acts as an energy source but also maintains a suitable osmolarity which is particularly significant for immature embryos (Liu *et al*., 1993). The optimum concentration of sucrose, therefore, varies with the stage of embryo development. Mature embryos grow well on media containing 2% sucrose but younger embryos need higher levels of the carbohydrate. This is in agreement with the fact that *in situ* the proembryos are surrounded by a fluid of high osmolarity that progressively falls as the embryo grows (Ryczkowski, 1960). Sucrose at 8-12% was found to be enough for the culture of proembryos of *Datura* (Rietsema *et al*., 1953) and *Capsella* (Monnier, 1978). When the osmotic pressure of the culture medium was synthetically escalated by adding enough mannitol along with moderate sucrose level (2%), it led to successful culture of proembryos of wheat (Fischer and Neuhaus, 1995).

4.4. Growth Regulators

Presence of growth regulators is not a prerequisite for normal embryo development. Moreover, their presence can lead to physical anomalies (Monnier, 1978).

Raghavan and Torrey (1963, 1964) reported that in *Capsella bursa-pastoris*, a balanced permutation of IAA, kinetin and adenine sulphate could make up for high osmolarity in the culture medium for successful culture of 55 µm long globular embryos. Suspensor can also be substituted by Gibberellic acid. Normal morphological and biosynthetic processes of embryogenesis can be ensured by adding ABA for culture medium (Fischer-Iglesias and Neuhaus, 2001).

Polar transport of auxins has been suggested to be involved in controlling cell divisions in the zone giving rise to cotyledons in globular embryos. When *Brassica juncea* globular embryos were treated with auxin transport inhibitors such as TIBA (N-1-naphthylphthalamic acid) *in vitro*, it led to formation of a ring-like structure, similar to fused cotyledons, around the shoot apex, in place of two separate cotyledons (Liu *et al.,* 1993).

4.5. Natural Plant Extracts

In *Datura stramonium*,Van Overbeek used liquid endosperm of coconut (coconut water) to grow embryos (200-500 µm), that could not be cultured on a definedculture medium. This indicated that CM contains an "Embryo Factor". Many researchers reported *in vitro* proembryo culture using endospermic or other plant extracts or even endosperm as the nurse tissue in many species. Alcoholic diffusates from young lupin seeds were found to support *in vitro* growth of 150 µm heart-shaped embryos similar to CM in *Datura tatula* (Matsubara1962).

5. PROEMBRYO AND ZYGOTE CULTURE

There is still no clarity about the nutritional requirements of very young embryos and zygote. Liu *et al*. (1993) were able to culture eight-celled, 35 µm long, early globular embryos of *Brassica juncea* effectively by employing a double layer culture system of Monnier (1976, 1978) comprising of two complex semi-solid media with different osmolarity. Till date, co-culturing with embryogenic microspores or feeder suspension cells dividing actively has been instrumental in successfully growing excised zygotes under *in vitro* conditions. Kumlehn *et al.* (1998) reported 90% regeneration of fertile plants from isolated wheat zygotes through direct embryo differentiation.

A technique was standardized by Kumlehn *et al.* (1999) for wheat where zygotes were captured in 100 µl droplets of 0.75% agarose and co-cultured with an appropriate embryogenic microspore population in liquid medium. This enabled direct observation of embryo development in 5-40 days post pollination. The first division of the zygote was symmetrical and it developed into a germinable normal embryo which formed fertile plants.

So far the success with excised zygote culture is restricted to monocots. In many dicots, *in ovulo* culture of zygote has been successful but excised embryos could be cultured only after the heart-shaped stage (Sauer and Friml, 2008).

6. GROWTH PREREQUISITES OF THE EMBRYOS

With regard to its nutrition, Raghavan (1966) identified two phases of embryo development: (i) *Heterotrophic Phase*- the early phase of development that may persist up to the globular or even a slightly later stage, during which the embryo obtains its nutrition from the endosperm and suspensor, and (b) *Autotrophic Phase*- this phase begins at the late heart-shaped stage when the embryo develops metabolic capability of producing substances obligatory for its growth and morphogenesis from basic mineral salts and sugar and draws upon its own metabolites. The decisive stage at which the embryo attains the autotrophic phase differs with the plant species (Raghavan, 1976). Even within the two

phases the external needs of the cultured embryo become simpler as it matures. This can be demonstrated in *Capsella bursa-pastoris* (Raghavan, 1966).The increasing independence of *Capsella* embryos with age is evident from the data presented in Table 1.

Table 1. Progressive nutritional independence during embryogenesis in *Capsella bursa-pastoris* (after Raghavan, 1966)

Developmental stage	Length of embryo (µm)	Nutritional requirement
Early globular	21-60	Could not be cultured
Late globular	61-80	Basal medium (macronutrients + trace elements + vitamins + 2% sucrose) + kinetin (0.002 mg L^{-1}) + IAA (0.1mg L^{-1}) + adenine sulphate (0.001 mg L^{-1})
Heart-shaped	81-450	Basal medium alone
Torpedo-shaped	451-700	Macronutrients + vitamins + 2% sucrose
Walking stick-shaped and mature	>700	Macronutrients + 2% sucrose

As reported earlier, the osmolarity of the culture medium has come up as a critical determinant for normal embryo growth. Decline in thickness, concentration, specific gravity and osmotic pressure of sugar and amino acid content of the ovular sap enveloping the growing embryo as the ovule aged was demonstrated in both monocots and dicots by Ryczkowski (1960-1972). Consequently, higher levels of the carbohydrates were needed for normal embryogenesis in younger embryos but 2% sucrose in the culture medium supported growth of isolated mature embryos successfully.

For optimal *in vitro* growth, the changing needs of developing embryos require their transfer from one medium to another after a certain period. An *in vitro* technique was given by Monnier (1976, 1978 and 1990) that permitted complete development of 50 µm long early globular stage embryos of *Capsella* which germinated in the same culture plate without any shift from their original location. Similarly, Fischer and Neuhause (1995) achieved normal direct embryogenesis of 100-160 µm long wheat globular embryos under in vitro conditions.

7. FUNCTION OF SUSPENSOR IN EMBRYOGENESIS

Suspensor is an appendage present at the radicular end of young developing embryos at globular to heart-shaped stage. The primary function of a suspensor is to provide nutrition to the developing embryos (*Phaseolus coccineus*, Yeung and Sussex 1979; *Capsella bursa-pastoris*, Monnier, 1984, 1990). It has been evidenced by number of researchers that presence of a suspensor leads to better growth and development of immature embryo (Bhojwani and Bhatnagar, 2008).

In vitro studies have revealed in the embryo that suspensor can be substituted by GA_3 in the medium. For instance, Alpi et al. (1975) had reported that when the embryo is at heart-shape stage, the GA_3 activity is highest in the suspensor of *P. coccineus*

8. THE PROBLEM OF PRECOCIOUS GERMINATION

There are different stages in the development of a zygotic embryo which have characteristic physiology and morphology. Under *in vitro* conditions, the immature embryos germinate prematurely, skipping further stages of embryogenesis and dormancy phase. This results into abnormal phenotypes which lack vigor.

Several treatments can prevent precious germination of immature embryos. This would include increasing the osmotic pressure of the culture medium by increasing the concentration of sucrose or

adding sugar alcohols such as mannitol, etc. or by using hormones such as ABA, which promote maturation (Dure, 1975).

9. APPLICATIONS

Following is an account of different basic and applied applications of zygotic embryo culture technique:

9.1. Basic Studies

The technique has facilitated understanding of basic processes of embryogenesis. It has led to deeper understanding embryo nutrition, physical and chemical factors affecting optimal embryo development function of various embryonal parts in its development, maturation and dormancy.

It has also led to deciphering of underlying physiology and genetics of embryogenesis (Fischer-Iglesias and Neuhaus, 2001; Schrick and Laux, 2001).

The technique has helped in *in vitro* manipulation of several crop plants, including legumes, cereals, cotton and a number of tree species and has helped in improvement of these crop plants.

9.2. Shortening of Breeding Cycle

The omission of the dormancy in zygotic embryo culture has curtailed the time required for carrying out breeding work in horticultural plants. For instance, Randolph and Cox (1943) demonstrated reduction of life cycle in *Iris* from two-three years to less than a year using embryo culture.

In soybean and sunflower, seed maturation takes 120-150 days. However, the life cycle of sunflower was reduced by half by *in vitro* culture of 10-day-old immature embryos (Plotnikov, 1983).

9.3. Rapid Seed Viability

Absence of dormancy in this technique has also facilitated detection of the viability of a particular batch of seeds, in much shorter duration of time. This test is considered more authentic than the staining methods for seed viability (Barton, 1961).

9.4. Propagation of rare plants

The technique can also be used for propagation of rare, expensive plants. De Guzman and Del Rosario (1974) exhibited the application of the technique in propagation of rare abnormal coconut phenotypes called Makapuno, which fetch better price. Such genotypes develop soft, fatty tissue in place of liquid endosperm. Using the technique of excised embryo culture, they raised plants from makapuno nuts, 85% of which bore makapuno nuts (De Guzman *et al*., 1976).

9.5. Haploid Production

Embryo culture has been applied for the development of haploids through distant hybridization followed by selective elimination of chromosomes. In the cross between *Hordeum vulgare* and *H. bulbosum*, double fertilization takes place but the chromosomes of *H. bulbosum* are specifically eliminated in the first few divisions of the embryo. Consequently, haploid embryos, with just one set of chromosomes of *H. vulgare* develop. However, these haploid embryos abort due to degeneration of endosperm after 2-5 days of fertilization. Complete haploid barley plants can be developed by excising and culturing the immature embryo. Wheat haploids are regularly produced by immature embryo culture after crossing it with maize (Laurie and Reymondie, 1991). Haploid plants of *Cucumis sativus* were developed by Lei *et al.* (2004) through pollination with irradiated pollen and subsequent embryo culture.

9.6. Genetic Transformation

Protoplasts derived from zygotes, that are able to regenerate into full plants, are perfect for marker-free transformation using microinjection. As it can avoid the toxic marker genes that have created higher apprehension about the safety of the edible transgenic crops, it is more appealing for these crops (Wang *et al.*, 2006).

9.7. Production of Rare Hybrids

Embryo abortion is a usual hitch in breeding programmes. Distantly related crosses are frequently futile even after normal fertilization as the hybrid embryo aborts on the mother plant due to the breakdown of normal endosperm development or embryo–ovular tissue incompatibility. In several such crosses *in vitro* culture of excised embryo (*ex ovulo)* has been very effective in rescuing the embryo and raising full hybrid plants.

The first successful interspecific hybrid produced with the aid of embryo culture was in the genus *Linum* by Laibach (1925). In these crosses the embryo was not able to grow to maturity *in situ* but when it was excised from the seed and cultured on nutrient medium it developed into a full plant. This work of Laibach set the basis of a method to surmount post-zygotic barriers to crossability where fertilization occurs normally. The technique has become a routine tool in the hands of plant breeders to augment the scope of hybridization to raise new genotypes.

Where embryo abortion occurs at an early stage and it is either difficult to excise and/or culture the embryo in isolation, *in ovulo* embryo culture (ovule culture) has been used to rescue the hybrid embryo.

Crosses between *Trifolium repens* and *T. hybridum* have failed by conventional as well as embryo rescue technique. Nevertheless, Przywara *et al*., 1989 could overcome this problem by removing the ovules 12-14 days following pollination and culture them for 5-6 days on a culture medium supplemented with 15% cucumber juice. Likewise, a number of hybrid plants were obtained from crosses between *Helianthus annuus* and *H. maximiliani* as well as *H. annuus* and *H. tuberosum* by Espinasse *et al*. (1991) using excised embryo culture subsequent to *in ovulo* embryo culture for a week.

10. SUMMARY

Zygotic embryo culture is commonly used as a standard *in vitro* procedure for the development of unique hybrids not possible by traditional breeding methods because of post-fertilization sexual incompatibility barriers involving the parental species. The use of this technique to develop hybrids was reported way back in 1925 when general tissue culture techniques and specifically embryo culture was not even optimized properly. In order to comprehend the nutritional and hormonal requisites of zygotic embryos belonging to various stages, impressive work on embryo culture was carried out in the 1960s and 70s. Presently, it is possible to save hybrid embryos aborting even at the globular stage, due to which the embryo rescue technique is integrated in plant breeding programmes routinely. However, culturing isolated *in vivo* and *in vitro* formed zygotes without supplementing the medium with a suitable nurse tissue has still not been achieved. Moreover, the function of nurse tissue still continues to be clearly defined. Zygotic embryo culture has developed numerous uses, such as reduction of breeding cycle, distant hybridization leading to haploid production, *in vitro* fertilization, rare plant propagation and comprehension of embryogenic developmental genetics (Bhojwani and Bhatnagar, 2008).