Embryo-culture and its use in plant breeding

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Introduction: In view of the growing significance attached to embryo-culture in recent years in experiments with plants, an attempt is made in this paper to describe in some detail the evolution of the embryo-culture and its achievements in the field of plant breeding, besides giving some newly recorded observations made by the author in his embryo-culture work in maize.

Embryo-culture defined: Briefly told, *embryo-culture* means culturing of excised embryos in an artificial medium and attempts at imitating nature, in that the essential food materials and conditions of growth that the embryo otherwise gets from within the ovule are artificially supplied to it. The term *embryo-culture* ordinarily refers to the culturing of mature or slightly immature embryos. Culturing, however, of very young embryos is termed *pro-embryo culture*. In a normal diploid seed the growth of the embryo and the endosperm go apace. It is found, that so long as the chromosomal complements of the different parts of a seed, namely, the embryo, the endosperm and the seed coat bear to each other a definite ratio, for example, 2 : 3 : 2 in a fertilized maize ovule, there is harmonious development for these different parts from the time of fertilisation to the final stage of maturity resulting in a normal fertile seed. But whenever this proportion gets upset as it occurs in a wide cross even after triple fusion, there is disharmony in the growth pace of these different parts. If the endosperm acquires a comparatively lower complement of of chromosomes than the embryo, than the growth pace of this is slower resulting in an arrest of the normal growth and subsequent collapse of the embryo. The term *pro-embryo* in a wide cross refers to the growth period of the embryo before this collapsible stage is reached.

The basic idea of embryo-culture was first conceived in Germany about 50 years ago. Hannig in 1904 in Germany grew embryos from *Raphanus* and *Cochleria* to maturity after they had been removed from their ovules when they were about 1·2 m.m. long (LaRue, 1936). Stingle in Germany in 1907 (LaRue, l. c.) grew embryos of several grains in the different cereals in the endosperm of other species. White in 1902 grew embryos of

Portulaca oleracea that were much smaller than those bred by Hannig and disproved the assertion of Dietrich in 1924 that embryos less than $\frac{1}{3}$ the normal size could not be grown in the culture medium (LaRue, l. c.). He could not, however, secure continuous growth for the embryo which stopped growing after 3 weeks. The growth of sweet cherry embryos in an artificial medium was reported by Tukey in 1933 and 1934. Carl D. LaRue 1936, grew immature embryos of both dicots and monocots to seedling stage in the culture medium. He failed, however, to grow an embryo less than 5 m.m. long into seedling stage.

The growth and development of the culture medium as it is in use today

(a) *Agar as base for the medium:* Since the time of Hannig agar media of varying concentrations have been in use for embryo culture. It has been used from $\frac{1}{2}$ to 10 percent. Although 10% has been found favourable with some in America, it is found that the embryos fail to develop in this as uniformly as in low concentrations. Half and one per cent have given good results and 0·6% generally speaking, appears to be ideal. At this concentration, there is enough water available which is necessary to support a growing embryo, at the same time, possessing sufficient degree of viscosity for the medium to support the embryo on the surface.

(b) *Salt content of the medium:* Various salt solutions named after their advocates, have been in use. Pfeffer's, Knudson's modified solution, Upanski's, White's, Crone's modified solution, Knopp's complete nutrient solution, Tukey's and Randolph's are some that may be mentioned. All these have more less the same complement of mineral elements, but in different forms and proportions. Tukey is of the opinion that there is no appreciable difference in effect between the different salt solutions used by various workers. The concentrations could be varied from 1 to 10 times with no appreciable effect upon embryos or seedling development so long as the concentration did not exceed that which is toxic to the plant (Tukey, 1934). Tukey's basic medium has KCl, $CaSO_4$, $MgSO_4$, $Ca_3(PO_4)_2$, $Fe_3(PO_4)_2$ and KNO_3 for its ingredients and is now widely in use for embryo culture work. One of the advantages of Tukey's mixture is that the salts may be ground, thoroughly mixed and placed dry in a stoppered bottle and used over a period of months. The solution used by Randolph is essentially that of White, excepting that instead of KH_2PO_4, he used Sodium-hexa-metaphosphate $(NaPO_3)_6$. It is prepared in two stock solutions

and mixed together at the time of use. $(NaPO_3)_6$ forms a soluble complex with both iron and calcium and eliminates the difficulty inherent in most other mixtures in retaining in solution the small amount of iron necessary for optimum growth (Randolph, 1945).

(c) *Sugar in the medium :* Sucrose, Glucose and Fructose have been in use in varying quantities. Sugar has been found necessary for inducing chlorophyll development in the embryo in the very early stages, as otherwise, it does not grow. Therefore, the strength to be used depends upon the stage of development of the embryo to be cultured. Growth of more fully developed embryos is inhibited by sugar. Preferences for the particular form of sugar appear to vary with different plants. For a general purpose medium, to cover the range of requirements of both young and old embryos, 5% sugar as sucrose appears to be optimal.

(d) *Growth promoting substances :* The utility of growth promoting substances in embryo-culture was recognised as early as in 1922 by Knudson. Heteroauxin, indolacetic acid, glycocoll, propionic acid, adenine, thiamine, ascorbic acid, succinic acid, nicotinic acid, panto-thenic acid and Vitamin B_6 are some of them in common use. Natural extracts from Canna tubers, Carrot, Garden beet, Coconut meal, Datura ovules, Almond meal and Yeast have been found to help embryo growth. Van-Overbeek (1941) found in coconut water, an active growth promoting substance. He found that young Datura embryos were particularly responsive to coconut water. He tried the growth of a 10–day–old Datura embryo measuring less than 0·5 m.m. in Tukey's general purpose medium to which were added some of the physiologically active substances indicated above in certain proportions fixed on a purely arbitrary basis (Van-Overbeek, Conklin and Blakeslee, 1942). The embryo failed to grow. However, when coconut water was added to this, there was better growth of the embryo. In 1942, Van-Overbeek succeeded in extracting embryo factors from coconut water by fractionation process. He showed for this fractionation product, an embryo activity in a dilution of 1 : 4,000 parts, compared to 1 : 110 parts for untreated coconut water. With the help of this he was able to grow a 0·45 m.m. long embryo several times more than by using cocoanut water as such. But roots did not develop, evidently due to a root inhibitor. This was removed by further fractionation. The new preparation showed a factor activity at a dilution of 1 : 19,000. Using this he was able to grow an embryo 0·45 m.m. long into a perfectly normal seedling (Van-overbeek, 1942).

Van-Overbeek and his associates were able to culture very young embryos of Datura aged ten days and measuring only 0·15 m.m. long into normal seedlings with these fractionation products.

Uses of Embryo-culture: The uses of embryo-culture in plant breeding can be considered mainly under the following three heads,

(a) *Culturing of slightly immature embryos:* Culturing of embryos removed out of slightly immature seeds, helps to grow readily, seedlings from such seeds that otherwise do not germinate when mature, until after the dormant period (Goff, 1900).

(b) *Culturing of mature embryos:* Culturing of embryos excised out of mature seeds, helps quicker germination in seeds that have hard and impermeable seed coats.

(c) *Embryo-culture in the pro-embryo stage to get up F-1. generations out of incompatible crosses:* In a wide cross the embryo sometimes grows normally but the endosperm surrounding it ceases to grow or grows at a much slower rate so that the normal growth of the embryo gets arrested. Embryo-culturing just before this happens, in other words, in the *pro-embryo* stage, makes it possible to grow the embryo into a seedling. By this method Blakeslee working with *Datura stramonium* as one of the parents succeeded in securing species hybrids from combinations which had given only a single viable seed from many hundred pollinations (Blakeslee, 1944). In Blakeslee's own words, "we no longer have to wait for the chance hybridisation between species and the later rare spontaneous doubling of their chromosomes in order to secure such superior varieties. With the use of colchicine we can now make this to order, provided we have the sterile hybrids to start with. Thus embryo culture method should considerably increase the source of these sterile hybrids".

Some new lights on certain aspects of embryo culture as conducted and indicated below by the author in its use and application to breeding in maize.

(a) *Culturing of pro-embryos normal of diploid maize aged 3—7 days;* Previous workers on maize reported that 10-day-old maize embryos over 0·3 m.m. in length grew steadily in the culture medium (Haagen-Smit, 1945). They did not, however, indicate the reaction in a culture medium of a pro-embryo less than 10 days old. In the present experiment, culturing of pro-embrys aged 3—7 days

in Tukey's basic medium containing active growth promoting substances did not help to grow them into seedling stage. None of the embryos excepting the 7-day-old one showed any signs of growth in the medium (Uttaman, 1949). The 7-day-old embryo grew but ceased growth at the end of the 5th day. A similar case has been reported by White in 1932 (LaRue, l. c.). He was able to grow an embryo of *Portulaca oleracea* measuring only 0·12 m.m. to a size of 1·84 m.m. by adding a fibrin digest to his culture medium. The embryo, however, was unable to grow further at the end of the third week. It is clear that these active substances are not able to fully supplement the deficiency of natural hormones that this needs for its full development.

(b) *The effect of cocoanut water on the growth of immature embryos of maize :* In this experiment, it is found that cocoanut water has a decisive depressing effect on the growth of a 2-week-old maize embryo, although it does not totally inhibit its growth in the culture medium. Van-Overbeek in 1941, reported that in Datura embryo the roots did not develop in the presence of cocoanut water but the shoot did. This finding is not, then, in absolute agreement with the above results in maize (Uttaman, 1949 [2]).

(c) *A study in contrast of the effects of cocoanut water on the growth of immature embryos of maize when applied before and after germination of the embryo :* In this experiment it is indicated that the cocoanut water does help the growth of a maize empryo when applied after the embryo has started growing rather than when applied before germination, although Haagen-Smit (1949) reported no effect for cocoanut water on this. The reason for this differential behaviour in the embryo-growth in the present experiment may be found in the hypothetical suggestion that by the time the embryo starts to germinate the embryo factors decompose into certain toxic component parts which depress the germinating embryo and that most part of the opportunity to benefit by the embryo factors, is lost to it. That the loss of emryo factor activity of these natural extracts due to standing, heating, chemical actions etc., may be due to a release of toxic substances by their decomposition has been demonstrated by previous workers on Datura embryos (Van-Overbeek, Conklin and Blakeslee l. c.). It is further indicated that the embryo active property of any natural extract could be more readily and clearly understood by its application to the embryo after the latter has been initiated into sprouting than by application before germination (Uttaman, 1949 [3]).

(d) *Growth promoting factors in corn germ extract:* Lampe and Mills (1933) have reported the growth of 10-day-old embryos of maize in agar containing mineral salts, dextrose and extracts of young corn ovules. Aqueous extract of 2-week-old corn ovules was tried by the author without any spectacular effect on equally aged corn embryos. In this experiment, extract of sprouting embryos of mature corn seeds is tried on young corn embryos. The maize germ extract was prepared by finely macerating the germinal embryos of maize and then sterilising by Seitz-filtering under vacuum pressure as the extract may not be stable to auto-claving. The results of the experiment show that the maize germ extract has a marked beneficial effect, much more than of cocoanut water on the growth of a very young embryo of maize (Uttaman 1949.)

(e) *Embryo-culture to obtain F-1. plants out of incompatible crosses in maize:* Wide crosses usually do not set mature fertile seeds. Occasionally, one or more partially filled fertilised ovules are met with. These respond to embryo culture when young, although the mature seeds show indifferent germination. In the above experiment, 66 partially filled seeds were obtained from a cross between a tetraploid and a diploid maize plant. These seeds when pot sown gave only 15 P-1 plants of which one was a triploid. This was crossed to a tetraploid and the three partially filled seeds obtained from this cross were embryo-cultured when 16 days old. All the three, confirmed to be beteroploids by root tip studies, were reared into young seedlings. Embryo culture of very young seeds in this way helps to secure a greater percentage of F-1 plants out of incompatible crosses than by the ordinary method of germination of these seeds when ripe, by pot sowing (Uttaman, 1949 [5]).

(f) *A preliminary investigation into the viability of immature embryos of maize under conditions of cold storage at freezing point:* In any investigation requiring the dissecting out of several hundreds of embryos from immature kernels, any devise that preserve the young embryos inside the kernels from loss of viability should have a special significance, in that such an operation could then be conducted through several days without being obliged to do it all at one moment. From the present investigation, it is found that the viability of the young embryos of maize aged three weeks, could be preserved through a period of about $1\frac{1}{2}$ months by keeping under cold storage at freezing point (Uttaman, 1949 [6]).

Culture technique: The embryo-culture technique mainly concerns itself with careful excising of the embryo from the seed and ensuring freedom from contamination by bacterial and fungoid infection of the embryo to be cultured.

The excising of the embryo out of a mature seed is best done after softening the seed coat by soaking for a day or two in water. If the size of the seed happens to be very small as in the case of a tomato seed, the operation can with advanage be effected by holding the seed between the edges of two mounting slides and then by dissecting with a fine razor blade. In the case of a very young immature seed, the excision is best done with the help of a fine needle and a scalpel under a preparation microscope. Employment of a fluoroscent light is advisable as the use of an ordinary incandescent light often seriously impairs the embryo, causing it to shrivel and die by the radiating heat. Freedom from contamination is gained by complete sterilisation of the culture medium and the containers on the one hand and on the other, by properly disinfecting the seed prior to the excision of the embryo, done under strict aseptic conditions. Sterilisation of the containers is best done by first cleaning the bottles and the caps with cleaning solution and then with boiling water. Screw caps are better than corks or plugs. After filling the bottles with the medium the caps are screwed half way down and the bottles auto-claved at 15 lb. pressure for 15 minutes. Upon removal from the auto-clave the bottle are cooled down by a slow process. This can best be secured by protecting the bottles from the wind current with a sheet of paper used as a screen. Otherwise there may be rapid cooling and the water vapour inside the bottle may condense down into water which might seriously affect the consistency of the medium. The medium may not set even after cooling. As regard the disinfection of the seed prior to the excision of the embryo, calcium hypochlorite method of Wilson has been in use for a long time 10 grms. of calcium hypochlorite is throughly shaken with 140 c.c. of distilled water and the clear liquid decanted. The seed is kept in this solution without bad effect for 3 minutes or more. Keeping for 5 minutes in a 2% chlorine solution has also been a standard practice.

The embryo-culture technique has now been greately simplified and standardised (Randolph, 1945). Any well lighted room reasonably free of the spores of moulds and bacteria is suitable for the transfer of embryos from the seeds to the culture bottles. The possibility of contamination from the air-borne spores may be reduced

considerably by spraying the table and the walls of the room with a 1% aqueous solution of phenol to which a few drops of a wetting agent such as Turgitol has been added.

For excising embryos out of mature seeds, the seed is sterilised in hypochlorite solution and then soaked in distilled water for 3 or 4 days according to the hardness of the seed with a daily change of water. Before excision the seed is dipped in 70% ethenol. Similarly all the instruments used in the operation and also the finger tips are dipped in this solution. The needle is then taken by a single passage through the flame of an alchohol lamp and then dipped in S. T. 37 (Hexyl-resorcinol) diluted 1 : 1 with distilled water. The embryo is like-wise dipped in this solution before taking to the medium.

The embryos are cultured first in darkness at 28 – 30°C. for 3 – 5 days and then transferred to weak day-light for another 3 or 5 days. There after the culture bottles are removed to the green-house from which direct sun light is avoided and in which the temperature is maintained at 65 – 85°F. After 2 or 3 weeks when the roots have developed, these are transferred to pots containing sterilised soil.

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A short resume of crop and plant protection, entomology-its past, present and future *

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Out of the seven lakhs of insect species estimated to exist in the world, about 10,000 are known to affect plant life. Of these about 500 species of insects are major pests affecting cultivated crops. As far as South India is concerned, nearly 600 insect species have been recorded as having a close relationship with cultivated plants and among these about 200 species are pests. It is with this number that the Crop and Plant Protection Officer (Entomology) has to tackle and devise remedial measures that would satisfy the agriculturist and horticulturist.

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