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NEW HYBRIDS FROM INCOMPATIBLE CROSSES IN DATURA THROUGH CULTURE OF EXCISED EMBRYOS ON MALT MEDIA[1]

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INCREASINGLY of recent years it is becoming possible to control the activities of the living plant by chemical treatment. The success in doubling chromosomal numbers with colchicine and other stimuli naturally led to an attempt to halve the chromosomal number by some similarly simple treatment. The fact that over two hundred haploids (1*n*) had turned up spontaneously in our cultures of Daturas since 1921 showed that the plant is capable of producing individuals with half the normal 2*n* chromosomal complement. Preliminary attempts to induce the production of 1*n* offspring by treating the unfertilized egg cells with a wide series of stimuli were entirely unsuccessful. In the summer of 1940 the cooperation of Drs. J. van Overbeek and Marie Conklin was secured in a more intensive attack on the problem. Something was learned about the processes involved in embryo development,[2] but none of the stimuli tested induced the production of 1*n* embryos. In the summer of 1941 they attacked the problem from a different standpoint in order to learn more about the factors involved in embryo development and attempted to dig out the young embryos and cultivate them on artificial media. The older embryos could be thus readily cultivated, but the smaller ones (under 0.5 mm in *D.*

[1] Read before the American Philosophical Society, November 19, 1943. Contributions from the Department of Botany, Smith College, New Series, No. 12. This investigation was supported in part by the Carnegie Institution of Washington and by a grant from the American Philosophical Society. The authors are indebted to Margaret Conlin, Jean Cummings, Susanne McLean and Mary Sanders, who as graduate assistants have made a large proportion of the dissections.

[2] J. van Overbeek, M. E. Conklin and A. F. Blakeslee, *Am. Jour. Bot.*, 28: 647–656, 1941.

stramonium) would not grow on the artificial media. Since in some cases the spontaneous (1*n*) haploids which appeared in our cultures had been shown to be twins developed within the same seed envelopes as the normal (2*n*) diploids, it was suggested that in such cases some substance in the normal twin might have stimulated the development of the 1*n* partner and that it might be possible to extract this substance and use it in the artificial media. Dr. van Overbeek suggested rather the use of coconut milk, which is a natural endosperm. With this it was possible to culture stramonium embryos as small as 0.2 and 0.15 mm in diameter.[3] Later with the cooperation of Dr. Haagen-Smit the "embryo factor" in coconut milk was concentrated and freed from a number of contaminating substances which deleteriously affected embryo growth.[4]

In reporting this work[5] it was suggested that such growth of excised embryos on artificial media might ensure the success of many wide crosses hitherto impossible. We now have evidence that this suggestion was well founded and we are utilizing the technique just described in a study of speciation within the genus Datura. To analyze chromosomal changes in evolution of the 10 herbaceous species of this genus, hybrids are necessary in which chromosomes may be matched up and compared. Unfortunately, hybrids between certain species have not heretofore been possible. Some of the blocks to crossability may be mentioned:

(1) Racial differences, presumably due to genes, may prevent hybridization. Thus, the first race of *D. leichhardtii* from Australia crosses readily with *D. discolor* as the pollen parent, but the second race of *D. leichhardtii* from the same country, which is indistinguishable in appearance from the first, gives only arrested embryos when similarly pollinated.

(2) The pollen applied to the stigma of the female parent may fail to germinate. This is not true, however, of pollinations between any of the ten herbaceous species of Datura. Lack of pollen germination does not seem to be an important block to crossability. Pollen of other genera of the Solanaceae, such as Petunia and Nicotiana, for example, have been found to germinate on stigma of *D. stramonium* and to ensure the setting of small but sterile capsules.

(3) The pollen tubes may burst before reaching the ovary and thus the opportunity for fertilization prevented as has been shown by an earlier study of Buchholz[6] *et al.*

(4) The slower growth of pollen tubes of short-flowered species may be a handicap to them in reaching the ovaries of long-flowered Daturas before the styles become withered. Perhaps such crosses could be facilitated by splicing of styles which Buchholz and Doak[7] employed to increase pollen transmission of extra chromosomes in *D. stramonium*.

(5) Fertilization may fail to take place after the pollen tubes reach the ovary. Whether this condition occurs in species crosses in Datura is not known, but not all the incompatible species crosses in the genus have been studied from this standpoint.

(6) Fertilization may occur, but the zygotes abort at an early stage. Thus, in the cross *D. stramonium* × *D. metel*[8] there are abundant fertilizations, but the fertilized egg cell develops to not more than 8 cells before the proembryos become aborted.

(7) Fertilization may take place and embryos develop to a later stage when something arrests their further growth and the imperfect seeds which result are incapable of germination. Of arrested embryos there are two main classes: those represented by the cross *D. inoxia* × *D. discolor* in which a large proportion of the embryos reach a relatively advanced stage before becoming arrested and those represented by the cross *D. inoxia* × *D. stramonium* in which only an occasional embryo in a capsule reaches such an advanced stage.

It is possible to dissect out the hybrid embryos which become arrested after the proembryo stage and to cultivate them on artificial media with the technique developed by van Overbeek and Conklin for normal embryos of *D. stramonium*. The media used contained the necessary salts, vitamins and the "embryo factor" secured from coconut milk. When embryos are small (diameter under 0.5 mm), only slight if any growth occurs without the "embryo factor." We have recently found that powdered malt extract can replace the "embryo factor" from coconut milk if sterilized by filtration instead of by heat.

With the malt media we have gotten growth of hybrid embryos from 11 new species combinations in Datura. By similar embryo culture we have secured species hybrids from combinations which had given only a single viable seed from many hundred pollinations. In addition, the dissection method has enabled us to secure numerous seedlings of certain species the seeds of which without treatment give a germination of only about 0.1 per cent. One aberrant species (*D. ceratocaula*), which is semiaquatic and

[3] J. van Overbeek, M. E. Conklin and A. F. Blakeslee, SCIENCE, 94: 350–351, 1941; *Am. Jour. Bot.*, 29: 472–477, 1942.

[4] J. van Overbeek, *Cold Spring Harbor Symp. Quant. Biol.*, 10: 126–133, 1942.

[5] A. F. Blakeslee, *et al.*, *Carnegie Institution of Washington Year Book*, 40: 214–225, 1941.

[6] J. T. Buchholz, L. F. Williams and A. F. Blakeslee, *Proc. Nat. Acad. Sci.*, 21: 651–656, 1935.

[7] J. T. Buchholz, C. C. Doak and A. F. Blakeslee, *Bull. Torrey Bot. Club*, 59: 109–118, 1932.

[8] Sophia Satina and A. F. Blakeslee, *Bull. Torrey Bot. Club*, 62: 301–312, 1935.

grows in shallow ponds in Mexico, had never hybridized with any Datura. Through use of malt media, however, it has given hybrids with several species among which is *D. stramonium*. In this latter species there have been developed a large series of chromosomal tester races (Prime types) with which the chromosomes in *D. ceratocaula* may be determined. Perhaps our most surprising success of the dissection method is securing hybrids between *D. inoxia* and a tree Datura. This suggests the possibility of extending chromosomal analysis to the group of tree Daturas which by some taxonomists are considered generically distinct from the herbaceous Daturas and to be included in a separate genus, *Brugmansia*.

The excised embryos may be extremely slow in developing in the culture media and for some time after surviving the shock of transplanting into soil, which is often a disastrous experience. Ultimately, however, the hybrids which survive have developed into vigorous plants without sign of weakness.

We have apparently succeeded in getting a new hybrid of Iris (*I. pseudacorus* × *I. versicolor*) from dissection of arrested embryos sent us by Dr. George M. Reed, of the Brooklyn Botanic Garden. We have evidence that the technique described may be of value also in other genera in securing new hybrids which have hitherto been impossible. Moreover, Skirm[9] has recently succeeded in getting a number of new hybrids from the dissection of hybrid embryos of different species of Lilium which appear not to need the aid of the "embryo factor" from coconut milk or malt extract for their development in artificial media.

Systematic study of other genera would be necessary before one would be warranted in taking the genus Datura as typical of the proportion of new hybrids which can be secured in other groups by cultivation of excised embryos. Our experience with Daturas, though incomplete, would suggest alluring possibilities with other forms. With the 10 herbaceous species of Datura there are 90 hybrid combinations theoretically possible if each species is considered separately as male and female parent. The formula is $n(n-1)$. Viable hybrid seed was secured in 18 combinations, a number which might possibly be slightly raised if a large number of pollinations had been made in some of the combinations. In 49 combinations no attempt has yet been made to dissect out arrested embryos or the capsules from recent pollinations have not yet reached a dissectable stage. In 12 combinations dissections have been attempted but without success. In 11 combinations, arrested embryos have been dissected and cultured. Of the 23 combinations actually tested 11, or 48 per cent., have given dissectable embryos which have been brought into cultivation. In attempting to get hybrids from arrested embryos, we have begun with those combinations which would be of most value to our study of chromosomal differences between species, but there is no reason to believe that when the combinations which have failed to yield viable seeds are completely analyzed, the percentage of arrested embryos which can be cultivated will be greatly altered. If we should succeed in our attempts to coax development of the early-aborting embryos, such as those found from the cross *D. stramonium* × *D. metel*, we might be able to secure nearly all the hybrids theoretically possible from the 90 species combinations among our 10 herbaceous Daturas.

The dissection technique may aid in an understanding of the factors involved in chemical regulation of embryo development and new hybrids which it seems capable of supplying may prove useful in genetical analysis of other forms than Datura. They give promise, moreover, of becoming of considerable value economically. Hybrids, especially from wide crosses, are characterized by increased vigor as exampled by the high yields of hybrid corn. Although intermediate forms are common, hybrids may be classified into fertile hybrids and sterile hybrids. The latter are sometimes called "mule plants" since they are like the sturdy mule in that they can not form sex cells since the chromosomes of one parent are too unlike those of the other parents to mate and form pairs; and pairing of chromosomes is necessary for sexual reproduction. Such sterile hybrids may be rendered fertile and pure-breeding with retention of hybrid vigor by doubling chromosome number.[10] In a number of cases sterile hybrids between species have been made fertile through spontaneous doubling of chromosome number which had taken place before recorded history to produce pure-breeding races which had been preserved by prehistoric man because of their superior qualities. Among such fertile "mule plants" may be mentioned our best varieties of wheat, oats, timothy, tobacco and cotton. We no longer have to wait ages for the chance hybridization 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 them up to order, provided we have the sterile hybrids to start with. The dissection technique should considerably increase the source of these sterile hybrids. These two methods in combination bid fair to be of considerable service in increasing the yield of plant products in a time when need is felt for ways of growing two blades of grass with the labor formerly required to grow one.

[9] G. W. Skirm, *Jour. Hered.*, 33: 211–215, 1942.

[10] A. F. Blakeslee, *Amer. Jour. Bot.*, 26: 163–172, 1939.

In this relatively new field there is opportunity and need for many investigators to explore the range within which artificial cultivation of hybrid embryos is feasible, to improve the technique of such cultivation and to adapt it to the different forms investigated and to study the factors which limit crossability with the aim of still further increasing the possibilities of hybridization.

It should be stated that others have also cultivated hybrid embryos on artificial media. Our contribution to the hybridization problem lies in the use of the "embryo factor" and the application of the technique of van Overbeek and Conklin which enable embryos to be excised and cultivated at an earlier stage, at least in Datura, than has heretofore been possible, and in the use of malt extract as a more convenient source of the "embryo factor" than coconut milk.

Further study of regulatory factors involved in fertilization and embryo differentiation should lead to a conscious control of a wider range of life processes.

USING ELECTRONS FOR MICROANALYSIS

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THE past decade has seen an exceedingly rapid growth of the development and use of electronic tools in all sciences. One of the most spectacular of these is the electron microscope which, with its extremely high resolving power, is able to project man's vision further than was ever before possible towards the direct observation of the building blocks of nature. While our vision has been greatly extended and now enables us to see things in the size range 20 to $10{,}000 \times 10^{-7}$ millimeters, the type of information that we obtain through the sense of vision has not changed in any way; that is, we see the size, shape and structure of the finer details of a specimen but nothing else. Such information is sufficient for the solution of innumerable research problems but it does not represent in any way a complete exploitation of the electron microscope or of the electron optical method. If we are to make full use of what we observe with the electron microscope we must supplement it by the proper application of controlled experiments or by the development of new tools which provide additional information in this same size range.

One of the most specific types of information that would be of immediate use in the field of electron microscopy is that pertaining to the nature or composition of the entities observed. In a large part of the work being carried on with the electron microscope pure samples are used so that the information with regard to shape, size and structure can be tied up immediately with the chemical and physical properties of the material. However, when the electron microscope is applied to problems in which complex or organized structures are present, the different entities observed are spread out in the image and there is no direct way of correlating the composition of a specific particle with a chemical analysis made on the specimen in bulk. A number of methods of attacking this problem have already been suggested and some preliminary work has been done.

One method is the correlation of the intensities of image points with the mass densities of corresponding object points. While the theory of the electron microscope shows that there is a relationship between the mass density of the object and the intensity of the image, it is difficult to obtain an accurate measure of density in this way because of the necessity of knowing accurately the thickness of any given object point. Another discouraging feature of this method is that more often than not intensity anomalies occur in the image as a result of electron interferences which take place if the particles being investigated are crystalline.

Electron diffraction has always been a very useful method of analysis—particularly of powdered chemicals. Unfortunately, like most other methods, it gives an analysis of the whole specimen. Recently, there have been attempts to apply electron diffraction to point analysis. In these methods the irradiating beam is confined to the small areas of the specimen under investigation. While this method shows some promise, it can be applied only in the case of crystalline particles and even in this its usefulness is limited by the fact that the individual particles are almost invariably single crystals. This requires that they have the proper orientation in the specimen before a pattern can be obtained and the pattern produced is that of a single crystal which in itself is not always sufficient for the identification of a compound. The third method which may be applied for identifying the chemical nature of particles is the use of specific reagents which change the mass density of the particles in question. While this method appears to be tedious and not too certain of success in any particular case, some exploratory work in connection with the selective staining of bacteria has been done.

Recently, in a paper to the *Physical Review*, J. Hillier of this laboratory has described a more direct electronic method of attacking the problems outlined above. The paper described a new type of instrument which is named the electron microanalyzer and in