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# ANTHER-CULTURING AND AGRICULTURE — PROBLEMS AND PROSPECTS

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The technique of anther-culture has triggered off a spate of activity in several laboratories of the world towards the rapid application of this method to the large-scale production of haploids. So far, this technique has worked very effectively in the case of a few crops, such as tobacco, *Datura,* and, to a limited extent, rice, wheat, *Petunia, Atropa,* and some species of *Solanum.* During the ten years that the anther-culture method has been in existence, a large volume of data has accumulated on the precise pathways of pollen embryogenesis, the factors controlling the response, the methods of chromosome doubling of the haploid phase and more recently, a method of inducing haploids directly from an isolated pollen-grain has also been developed.

As regards the mode of haploid formation in anther-cultures, two types of development have been observed: (i) where the pollen-grain directly produces an embryo which develops into a haploid plant as in *Nicotiana* and *Datura.* and (ii) the pollen produces first a callus, from which plants are regenerated, Although the first pathway would be ideal, since we would get exclusively haploids, the second one might be desirable, especially in species where the response of anthers is poor, since the few pollen calli could be further multiplied to give more tissue from which plants could be regenerated. Rice, *Petunia, Solanum,* tomato, *Lolium* and several others come under the latter category. From the plant breeders' point of view, since the majority of plants regenerated from the pollen callus are homozygous diploids, the callus pathway is a welcome situation. Even the other aneuploids that result from the callus are not to be ignored, since in the case of sugarcane, disease-resistant clones have been obtained from callus-culture.

However, we face even greater challenges in the case of perennial plantation crops with long life-cycles. In them, haploidy induction through the conventional breeding techniques have been meagre. A vast scope lies ahead and it calls for intensive experimentation on anther and pollen culture in the case of woody perennials, such as forest trees, rubber, cocoa and other plantation crops, such as tea, coffee and the palms. Practically, no significant effort has gone into these difficult crops, In the case of tea, we have been able to induce a pollen callus; and in *Hevea,* too, callus has been obtained in anther cultures by a Ceylonese worker.

As regards the practical application of the anther-culture method of haploid production in crop improvement, the Japanese have taken the lead in releasing true-breeding stabilized lines of diploidized haploids in tobacco, combining high yield wih disease resistance. They are also ahead in rice haploidy work. The Chinese scientists have similarly exploited this technique in the case of wheat haploids and have produced stabilized lines.

Haploids as potent tools for basic genetic research are unrivalled and the anther-culture produced haploids of tobacco have been extensively used for

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inducing new mutants and in studies of recombination and somatic cell hybridization. Thus there is a very urgent need for a strong promotion of research on haploids and their utilization in our country with its vast resources of plant species and scientific talent.

## INTRODUCTION

Ever since the discovery of the first-ever haploid higher plant, as a spontaneous mutant in *Datura stramonium* by Bergner (Blakeslee *et al.*, 1922), conscious efforts to isolate as well as artificially induce haploids have been going on in several crop plants, with varying degrees of success. Some of the techniques used include, distant hybridization, chromosome elimination, ionizing radiation, isolation of twins, genetic selection techniques, the use of alien cytoplasm, the pollen carrying a dominant marker and so on. However, none of these methods have caused so much stir as that of anther-culture discovered accidentally some ten years ago by two scientists at the Delhi University, Department of Botany, (Sipra Guha and Satish Maheshwari, 1964, 1966). While examining the cultures of immature anthers of *Datura innoxia* (2n=24) for their morphogenetic potential, these scientists observed plantlets emerging from inside the anther cavity, whose origin was traced back to the repeated cell divisions in the pollen grain, resulting in an embryo instead of the usual pollen-tube.

Today, this technique of anther-culture and the latest innovation of pollen-culture developed by Mrs Colette Nitsch (1974), of France, are engaging the attention of a large number of scientists the world over as the most potent tools not only for basic genetic research but also as a plant-breeding technology (Kasha, 1974). Some of the achievements already made in several annual crop species and the vast possibilities to be explored in the perennial and plantation crops will be discussed and the strategy for future research in this field will be outlined in this paper.

## MATERIALS AND METHODS

*Anther-Culture* : The crucial factor in the selection of the material for anther-culture is the high induction frequency. So far, no other species has matched the nearly 100 per cent response of the planted anthers in the case of *Datura* and *Nicotiana*. Another advantage, particularly in the case of *Nicotiana*, is the high recovery of haploid individuals through the direct embryogenesis of pollen as against the mixed population of haploids, diploids and higher ploidy plants in *Datura* (Narayanaswamy and Chandy, 1971; Iyer and Raina, 1972).

In the less responsive species, the critical factor would be the number of plantlets produced per anther, or the number of pollen calli from which plants can be regenerated subsequently.

The next vital factor is the stage of excision of an anther for culturing and it is now agreed for most species that the post-tetrad, uninucleate microspore stage is the ideal one. The extensive work of Nitsch and Norreel (1973) has provided the basic preconditioning factors for eliciting the maximum response in the anthers cultured. A traumatic shock given to the excised flower-buds of *Datura innoxia* by keeping them at 3°C for 48 hours greatly accelerates the rate of pollen-embryo formation in the anthers. In the case of rice, Wang *et al.* (1973) have enhanced the frequency of pollen-callus induction by pretreating the panicle for 48 hr at 10°C.

*Nutrient medium*: The experience so far gained over the past decade indicates that media based on Murashige and Skoog (1962) give reasonably good anther response in a variety of species, and supplements like coconut milk, casamino acids, yeast or malt extracts do enhance the rate of induction. The addition of auxins, such as I.A.A. and N.A.A., and kinetin has proved effective in the case of rice anthers, whereas *Datura* can respond in the absence of auxins, too. Sucrose is generally used at 2–3 per cent concentration for most species, although Ouyang *et al.* (1973) have obtained enhanced response in the case of wheat anthers, using 6 per cent sucrose. Experiments of Sunderland (1974) have established the crucial role of sucrose in providing the initial stimulus for pollen embryogenesis in *Datura innoxia* and *Nicotiana tabacum*. In our laboratory, we found that 3 per cent (w/v) bee's honey can replace sucrose in *Datura metel* anther-cultures for the induction phase, although the subsequent growth of haploids would need other supplements, too.

The initial incubation of anther-cultures in darkness has proved beneficial in our experiments. For the ultimate success of the technique, the post-culture nurturing of the haploid plants is the most decisive factor, and here the important thing to remember is to 'harden' the root-system in the medium itself or transfer it to a water-culture for a few weeks before planting in the soil. Mortality rates are usually high at this stage if not handled with sufficient care. Humidity seems to be quite a deciding factor, particularly because for most of the plants raised from cultures it becomes off-season, so that they have to be reared indoors, or in a well-controlled glasshouse with humidity sprays. The problem is rather acute with high-moisture-requiring species, such as rice.

*Pollen-culture*: Although isolated cultures of pollen-grains have been attempted long back in gymnosperms (Tulecke, 1953), the first comprehensive work on angiosperm pollen-culture is that of Nitsch (1974) in *Nicotiana tabacum* and *Datura innoxia*. The key factor lies in stimulating the uninucleate microspore to divide into two equal cells instead of the usual unequal vegetative cells (bigger) and the smaller generative cells, so that both now become 'androgenic'. Briefly, the pollen-culture technique involves the pre-treatment of the post-tetrad flower-buds at low temperatures (3–5°C) for 48 hr and the isolation of microspores through a suitable sieve, eliminating all the wall tissues of the anther. These pollen grains are 'sown' in 6-cm petridishes, containing 2.5 ml of a modified Halperin's medium (1965) to which a filter-sterilized water extract of anthers from one-week-old cultures has been added at a concentration of 5 anthers per ml. After incubating for 21 days at 27°C in a humid chamber, hundreds of haploid plantlets sprout in each dish. Nitsch (1974) has calculated that by using this method, one can obtain in tobacco, 7,200 plants from one flower-bud, of which 6,984 are haploid and 216 homozygous diploids.

## Results and Discussion

### *Basic Findings*

As a result of the extensive studies made in the Genetics Division of the IARI, New Delhi (Iyer and Raina, 1972), and at the John Innes Institute, UK (Sunderland, 1974; Sunderland and Dunwell, 1974), a clear picture has emerged with regard to the precise pathways of pollen embryogenesis in anther-cultures of *Datura* and

*Nicotiana.* The microspore nucleus can divide to give unequal daughter-cells, of which the bigger (vegetative) partner continues the embryogenic development, whereas the smaller one degenerates (*A* pathway); or, the first division results in two equal cells, both of which are embryogenic (*B* pathway). Alternatively, the two unequal cells of the [redacted]athway can both be embryogenic and contribute to the formation of pollen embryo (*C* pathway).

The second possibility is the formation of a pollen callus instead of a pollen plantlet, and this callus would naturally be a conglomeration of several pollen calli, which can be subcultured and multiplied. In *D. metel,* both direct plantlet formation and pollen callus have been obtained, whereas in the case of rice and several other crops, only a callus has been obtained by us and several other workers (Niizeki and Oono, 1968; Oono, 1975). The resultant plants from the callus are largely homozygous diploids, along with a small percentage of haploids, and higher ploidy plants. From the basic geneticists' point of view, it would be desirable to get only haploids, particularly for mutation (Carlson, 1970; Melchers, 1974) and somatic cell-fusion experiments (Carlson *et al.,* 1972; Melchers and Labib, 1974). However, since the induction frequency in most other species, particularly monocots and the perennial crops is of a low order, even the few pollen calli are welcome, as they can be further multiplied for regenerating plants. There is no reason to get alarmed at the chromosomal alterations frequently met with in callus culture which often result in plants of varying ploidy, since in the case of sugarcane, Krishnamurthi and Tlaskal (1974) have isolated a Fiji disease-resistant clone through callus-culture.

The other important basic data accumulated in anther-culture pertain to the precise pre-conditioning factors that elicit the maximum response from the anthers in artificial culture. Here, the work of Nitsch's laboratory in France (Nitsch and Norreel, 1973) is noteworthy, at least with regard to two species, *Datura* and *Nicotiana.* A cold shock given to the selected buds for 48 hr at 3°C before culturing, triggers off embryogenic activity in a greater percentage of anthers than without this pre-conditioning. Evidence of such effect is accumulating in other species of crop plants, e.g. rice.

The most exciting recent development has been that of Mrs Nitsch's elegant demonstration of a pollen-culture technique for the large-scale production of haploids in the case of *Nicotiana tabacum* and *Datura innoxia.* If this technique could be extended and made generally applicable to other valuable crop species, truly the plant breeder and the geneticist have struck a gold mine in biological research. With the fast tempo of activity now prevailing in several laboratories of the world, one is tempted to be optimistic that the pollen-culture technique would become a routine for haploid production.

## Results of Applied Value and the Future Possibilities

*Rice* : Oono (1975) has derived completely fixed uniform progenies of four pollen plants through the anther-culturing of the *japonica* × *indica* $F_1$ hybrids. One plant was carrying a single recessive short-culm mutation in the heterozygous state and another plant was homozygous for a chlorophyll mutation (*maculata*). The highest frequency of pollen-callus induction was 8 per cent in anther cultures of var. Norin 33. At the IARI, we have recorded the highest response of 15 per cent in the

pigmented var. *Crossa,* and 12 homozygous diploidized lines have been raised. They are now in the third ($A_3$) generation (Iyer and Raina, 1972; Raina and Iyer, 1974). Recently, Taiwan Chinese workers (Woo & Su, 1974) have also employed the $F_1$ anther-culturing in their *indica* (IR-8) and *Japonica* (Chianung 242-$d_3$) crosses, to overcome sterility and continuous segregation of the hybrids. The most intriguing fact in the case of rice is the enormous variability in response to different varieties (Guha *et al.,* 1970; Iyer and Raina, 1972; Guha-Mukherjee, 1973).

*Wheat and related genera* : Ouyang *et al.* (1973), of the Academia Sinica, Peking, have used anther-culture successfully to raise haploids from the $F_1$ hybrids and stable varieties or the $F_2$ hybrids of spring wheats, and obtained a uniform progeny of spontaneously doubled haploids. These stabilized hybrids possessed the white grain colour of the pollen parent (the female was red-grained) and significantly exceeded the parents in some agronomic traits, like grain number and grain weight of the main spike. Wang *et al.* (1973) of the same Institute have reported the successful production of pollen plants from the octoploid triticale. Kimata and Sakamoto (1972) have also reported success with the hybrid between *Aegilops caudata* × *Ae. umbellulata,* where they obtained haploid albino plants.

*Barley* : Although haploid barley plants have been produced through anther-culturing (Clapham, 1973; Bouharmont, 1974), the frequency of induction (3 per cent) is not consistent or high enough for a more general application of the method. On the other hand, the somatic chromosome elimination technique of Kasha and Kao (1970) is presently being used extensively for producing haploids in barley breeding in Guelph (Canada), Aberystwyth (Wales, U.K.) and Riso (Denmark). A comparison made by the Guelph workers (Park *et al.,* 1974) between the haploid method with pedigree and single-seed descent methods in barley breeding has revealed that the performance of 52 doubled haploid (*DH*) lines from each of the two crosses had genetic variability for yield and other agronomic traits similar to that of the material derived from conventional breeding. The stability of performance over locations showed no deleterious effects on complete homozygosity of *DH* lines, but the major advantages of haploid breeding were the time saved (3–4 generations) to obtain homozygous lines and the ease of selecting for desirable traits.

We must, however, remember that the pollen plants derived through anther-culturing are different from those derived from the *vulgare* × *bulbosum* cross in two respects : (a) they never had any contact with the egg cytoplasm, and (b) they had not had any contact with *bulbosum* chromosomes. These differences are of interest, since Nilsson-Tilgren and Wettstein-Knoweles (1970) have shown in *Nicotiana* pollen plants that the male plastome factors responsible for albino phenotype are present in the pollen at the time when the anthers are excised, i.e at the pollen-grain mitosis.

*Tobacco* : By far the most responsive of all higher plants tried in anther-culture is *Nicotiana tabacum,* in which case 45 per cent anthers produce embryos (Nitsch and Nitsch, 1969); 86 per cent response was obtained under strictly controlled conditions by Dunwell and Perry (1973) at John Innes Institute. The Japanese have been very quick in exploiting this technique for tobacco improvement of flue-cured var. MC 1610, for disease resistance (Nakamura *et al.,* 1974). They have obtained three promising doubled haploid lines, MCA-11, MCA-19, and MCA-27 from the

cross between MC-1610 and Coker 137, showing high resistance to bacterial wilt and black shank, with agronomic and chemical characteristics similar to those of MC-1610.

*Dioecious crops* : These crops allow sibbing only, which is a much slower method than selfing for producing inbred lines. Here, the monoploid method seems promising in the production of inbreds and hybrid cultivars. *Asparagus* anther-culture gives after diploidization 50 per cent homozygous YY males ('supermales') which when crossed with any female produce entirely male progenies. Homozygous females are produced from polyembryonic seeds by chromosome doubling of the haploid and through repeated backcrosses each homozygous female gives, at first an isogenic male, then a homozygous line reproducible through seeds. These lines are crossed among themselves to get homogeneous $F_1$ hybrids, several possessing heavy yield, and earliness. The commercial production of these hybrids is easy, since the parental lines are seed-propagated. This new type of the $F_1$ asparagus, characterized by great homogeneity could not be obtained without using haploids (Thévenin, 1974). Similar possibilities exist in the papaya and certain palms, and these need to be investigated.

*Vegetable and forage crops* : In *Brassica* and *Raphanus,* where self-incompatibility is being used in hybrid seed production, selfing is possible only through bud-pollination which is time-consuming and often erratic. Further, this forced selfing may impose a selection pressure for weakened self-incompatibility, making the lines useless as parents of hybrid cultivars. The obtaining of monohaploids through anther- or pollen-culturing and their subsequent doubling would lead very quickly to completely homozygous lines without the chance of lowering the level of self-incompatibility.

*Autotetraploid crops* : Potato, alfalfa and some grasses are hard to improve with conventional methods owing to their complicated tetrasomic pattern of inheritance. Haploidization would reduce them to the diploid level where possibilities exist of benefiting from all advantages of breeding at the diploid level. Theoretically, it looks very attractive to breed hybrid cultivars in the case of potato and alfalfa (Bingham, 1969), preferably double-cross hybrids because of their autotetraploidy, and here haploids may be used to obtain inbred lines. Once a superior double-cross cultivar is obtained, it can be multiplied vegetatively *ad infinitum.* It can also be recreated through seeds, since the parental lines also may be vegetatively maintained. Propagation through true seeds eradicates nearly all virus diseases of potato, which might make hybrid potatoes sown as true seeds and transplanted in the field, an attractive proposition for a developing country, like ours. Haploid plants have been induced in the case of potato by Dunwell and Sunderland (1973) through anther-culturing, and even tuberization has been obtained *in vitro.*

*Forest trees and plantation crops* : These long-duration perennials pose tremendous challenges to the breeder, and homozygous lines can be produced only through the haploid method, particularly in self-incompatible species, such as tea and cocoa. Since testing for combining ability is a long-drawn programme, it is all the more necessary to start the work immediately, so that our future generations can reap the harvest.

Although anther-culture attempts have been made in a number of woody perennials, so far only a limited response has been reported in a few species. Thus

callus has been induced in anthers of *Ulmus americana* (Brown and Sommer, 1974), and rooting calli from *Alnus tinctoria, Prunus apetala, P. edoensis, P. lannesiana* anthers, and in microsporangia of *Cryptomeria japonica* (Sato, 1971, 1972). Shoots were produced in the dark from the anther calli of *Populus sieboldii* × *P. grandidentata* (Sato, 1974), which rooted on transfer to light. Anthers of *Betula* and *Picea abies* (Huhtinen, 1972) also gave calli of uncertain origin.

Quite a few reports of callus induction from the pollen of several gymnosperms are available in the literature but none of these have yet been differentiated to give haploid plantlets. There are a few instances of embryo differentiation from megagametophyte tissue cultures of *Gnetum* (Vasil, 1963), *Zamia* (Norstog, 1965), and *Pinus* (Bonga, 1974).

As regards the rubber-tree, *Hevea brasiliensis,* there is a recent solitary report from Ceylon (Satchuthananthavale, 1974), of pollen callus induction from anther cultures of cultivars RRIC-52, and KH-400. No differentiation has so far been observed. Among other perennials, haploid pollen calli have been induced in tea (*Camellia sinensis* var. China) by Raina and Iyer (1974), in *Vitis vinifera* by Gresshoff and Doy (1974), and in *Prunus armeniaca* (Harn & Kim, 1972). The chief handicap in all these species is the slow growth rate of the calli and the lack of differentiation so far. This clearly calls for a more intense experimentation on these crops.

Another valuable tree species in the case of which haploids are being used for heterosis breeding is the cocoa, *Theobroma cacao* (2n=20), which is also self-incompatible. Dublin (1974) has already identified haploids with a distinct phenotype and produced homozygous diploids through colchicine. The scope for the artificial induction of haploids through anther-culturing is yet to be explored. Similar is the case with coffee.

## Strategy for the Future

Now that the scope for inducing haploidy through anther-culturing and pollen-culturing is fairly well recognized, it is now up to us to see that these methods become useful adjuncts to plant breeding. For a rapid application of these tools in crop-improvement programmes, it is essential that the tissue-culturist establishes a close liaison with the crop breeders and makes an extensive use of the $F_1$ hybrid materials for anther- or pollen-culturing, so that the derivatives can be screened for both qualitative and quantitative traits, such as disease or pest resistance, yield and quality as well as wide adaptability.

Even though the anther-culture technique was developed in India, we seem to have left the initiative to the Western and Far-Eastern countries not only with regard to basic research but also with regard to its rapid application to crop improvement.

Our programmes for the future should, therefore, be concerned with the immediate exploitation of the anther- or pollen-culture technique or of both in the case of crops in which a high induction frequency has already been demonstrated. As a first step, we at the IARI have already established a close contact with the workers at the Tobacco Research Institute at Rajahmundry. They have come forward not only to furnish the hybrid material but also to establish a unit for anther-culturing. The next step has been to initiate a collaborative programme in respect of rice

with the breeders at the IARI, the CRRI, Cuttack and the AICRIP, Hyderabad, for a large-scale attempt at anther-culturing to hasten their hybrid-rice programme. With regard to *Datura*, the workers at the Regional Research Laboratory of the CSIR, at Jammu, have already been induced to take up anther-culturing for the isolation of high-yielding types, since this work involves the screening of leaf-alkaloids in their laboratory.

The other crop, in the case of which a collaborative effort is under way is *tea*, which is posing problems of logistics at the present. However, if haploids can be produced effectively in the case of tea, it would not be hard at all to induce the Tea Research Stations at Jorhat, Coonoor or Palampur to set up facilities for anther-culturing at their stations, since the crop cannot be grown elsewhere.

In conclusion, it may be said that although anther-culturing has now come of age, research effort in the country of its birth has been microscopic and scattered. This is the time for all interested workers to get together and launch a vigorous, co-ordinated programme in selected crop plants in which an immediate demonstration of the practical utility of anther-culturing for producing haploids can be made. All the funding agencies for scientific research in the country should be more liberal in extending support to projects on haploidy and tissue-culture, so that more and more workers are encouraged to devote their energies to this exciting field. A national centre should be formed to co-ordinate this activity and to allocate areas of intensive experimentation, based on regional problems, and availability of material and facilities.

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