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MY ODYSSEY OF TWENTY-FIVE YEARS OF RESEARCH : FROM MORPHOLOGY TOWARDS MOLECULAR BOTANY*

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Mr. President, Professor Agarwala, Professor Murty, distinguished members of Botanical Society and friends :

At the outset, I should like to express my very deep gratitude and thanks to you all for the great honour you have done me by awarding this coveted medal in the memory of the late Professor Birbal Sahni. I regret I never met Professor Sahni—because of the age difference, a meaningful contact was impossible—however, I heard a great deal about him from my father who was a contemporary of his and also his colleagues at Lucknow in the thirties. In any case, as a student of Botany one cannot fail to be profoundly impressed by his classic studies. Indeed, when one recalls them—discoveries such as those relating to the Pantoxyleae, the Gondwanaland and Cycads—and compares one's own contributions one feels rather humbled. However, during the last 25 years I have had the good fortune of having many a hardworking and dedicated student and I accept this honour more as a recognition of their work rather than my own. Thus, in this address I shall largely deal with their researches.

Why the Word "Odyssey"?

The word 'odyssey' comes in the title because circumstances have buffeted me and my plans too often. As a high school student, I had planned to take up Chemistry for a career. But thanks to all the turmoil (especially on account of the partition, compelling a movement from one homeland already adopted in Dacca back to India), we landed up at Delhi rather abruptly in 1949. Under the strong and joint influence of both my father and Professor Johri, I found myself 'trapped' into doing not only a Botany Honours Course but also M.Sc. and a Ph.D. degree under the supervision of their watchful eyes. Since, in 1954, there were hardly any facilities for any kind of physiological or even tissue culture work, the problem assigned to me was the "*Embryology and Systematics of the Family Lemnaceae*". I am sure this family is known to every one here for its peculiar plants, like *Lemna*

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paucicostata and *Wolffia microscopica*, the latter being the world's smallest flowering plant. *Wolffia* is so bizarre that before flowers were discovered, it was once classified as an alga, and even after flowers had been discovered in this and other members, a controversy has remained about their phylogeny and evolution. According to one view (backed by Engler) the aroids gave rise to *Spirodela*, *Spirodela* to *Lemna* and *Lemna* to *Wolffia*—all by retrogressive evolution and dimunition, of plant size. On the other hand, just before I commenced my thesis work, the famous Belgian school of Martens came up with the idea that Helobiales, quite remote and another divergent branch of the monocot "phylogenetic tree", gave rise to the Lemnaceae.

My work on the embryology of the various Lemnaceae settled the question I think, decisively. The main points were as follows : *One*, although members of the Araceae have a monosporic embryo sac and most of the Lemnaceae have bisporic, the primitive duckweed *Spirodela* has a monosporic embryo sac, and thus there was an excellent case for the view that aroid-like ancestors produced *Spirodela*-like plants and that from the latter the remaining Lemnaceae were derived. *Two*, the endosperm is not only cellular in both families but a prominent haustorium characterizes both aroids and duckweeds, the similarity of endosperm of *Spirodela* to that in aroids being especially striking. As is common knowledge, all Helobiales have a quite different type of endosperm, the so-called Helobial type, where part of the endosperm is free nuclear to start with. The pattern of endosperm development sets apart the Helobiales and Lemnaceae put tightly knits the two families, Lemnaceae and Araceae. Unfortunately, it has not been easy for me to delve into the subsequent taxonomic literature in detail, but, so far as I know, this is the accepted scheme of things in all later works.

To Yale and Caltech

The desire to do some physiology and biochemistry nonetheless remained strong. And as soon as the opportunity came of the Smith-Mundt-Fulbright Fellowship in 1959, I applied for admission to various universities—the then meccas for research in plant physiology and plant biochemistry in the United States. Letters were duly sent to Professor Galston at Yale, Professor James Bonner at Caltech, Professor Thimann at Harvard and Professor Melvin Calvin at Berkeley (of the Calvin cycle fame). To add to my predicament and puzzle, I got acceptance from all. But, finally, I opted for Yale and Caltech.

It was a rewarding experience to be in New Haven and in the charming campus of Yale. Professor Galston introduced us (Mrs. Maheshwari and myself) to problems relating to mechanism of auxin action. After a year at Yale, we moved to Caltech at Pasadena and worked with James Bonner, the famous plant biochemist. For several reasons, the experience at Caltech was even more rewarding. Here, I came into contact also with Robert S. Bandurski who was spending a sabbatical in the Bonner laboratory. At Caltech both Mrs. Maheshwari and I participated in the work on discovery of RNA polymerase in plants. While Mrs. Maheshwari along with a Chinese colleague Dr. R. C. Huang concentrated on the pea enzyme, I worked with Bob Bandurski on tobacco. We are happy to have been involved in such work, because this was the beginning of all nucleic acid work on plants and which

has now led to the coming of age of the field of Plant Molecular Biology.

Coming Home—Research on Flowering of Duckweeds

But coming back home, I was again lost in a department without any radioactivity measuring instrument, or sufficiently powerful centrifuges, or other essential facilities. I found that no "fancy" work of the sort I imagined was at all possible. Nevertheless, some tissue culture facilities did exist in the department. And it seemed a good idea to return to the Lemnaceae, but now to grow these plants in aseptic cultures—for which they are so eminently suitable—and study their flowering, particularly because this had been thought to be so rare among these plants. I had also been influenced in research on flowering by my acquaintance in the USA not only with Bill Hillman, but the well-known plant physiologists, Hendricks and Borthwick. Not only were the plants easily cultured, but we showed that both *L. paucicostata* and *W. microscopica* are short day plants. Indeed, we were happy to have added these two plants to the small list of highly sensitive short day plants for flowering studies since both plants turned out to be sensitive to induction by even one photocycle. Moreover, flowering response could be observed as soon as 3 or at most 4 days of the photoinductive treatment. We have now worked on these plants for almost 20 years repeatedly confirming these basic features. Red light even of 1 minute duration completely inhibits flowering clearly indicating a phytochrome control. Nonetheless, some findings which are of more general, perhaps greater, interest is that cytokinins appear to be the "florigens" in these plants, which has become a fact of some 'textbook' importance. Earlier to our work, only gibberellins and auxins had been shown to have a florigenic role in plants : gibberellins in the long day plants and auxins in some day-neutral plants like pineapple. However, not much success had been achieved in inducing flowering in SD plants. The potency of cytokinins in induction of flowering is now demonstrated in practically all duckweeds. The work of a number of students, present and past, has pointed out a very special role also of two other groups of compounds. The first group comprises metal chelating compounds like EDDHA (ethylenediamine -di-o- hydroxyphenyl -acetic acid) and 8-hydroxyquinoline. EDDHA is known to be a very powerful chelating agent of iron and much unpublished evidence points to a rather specific role of iron in flowering. However, another extremely interesting group of compounds comprises salicylic acid (SA) and its close derivative aspirin which I am sure requires no introduction to any member of this audience. Both aspirin and SA have been known in plants for decades but until recently people did not know of any role in plants. Our work demonstrates that these compounds are very powerful in inducing flowering, exceeding even the potency of cytokinins. SA can also chelate ions, but it may well turn out to be a new hormone.

Cell and Tissue Culture-Discovery of Anther Culture Technique for Production of Haploids

As narrated above, various circumstances led me into photobiology. We also initiated some work on hormonal and biochemical aspects of reproductive development which was to some extent influenced by the main trend of research in this department. Apart from Calcutta,

for a while Delhi became a centre for analytical studies on extraction and identification of growth hormones, such as auxins, gibberellins and cytokinins. We were probably the first to introduce in India the technology for extraction and bioassay of cytokinins (again influenced by a brief acquaintance with Carlos Miller in U.S.A.) and we even reported in pumpkin and watermelon a new cytokinin. But due to various reasons, largely our inability to acquire sophisticated equipments like GC-MS which were clearly beyond our reach from the financial angle, this line was abandoned. But another area in which I developed a more abiding interest is cell and tissue culture and for which good facilities had been created in the department by early sixties. I have the feeling that when the Executive Council of the Botanical Society decided to select me for this award, the discovery of anther culture technique raising haploids might have been the deciding factor. This discovery has an interesting history of its own. I hope that I do not shock any one in the audience by saying that despite my father's great research interest in the department on methods of inducing the formation of haploids, I was not in the least enthusiastic about it. The reason was that Mrs. Nirmala Maheshwari had already a hard time tyring to induce haploids in unfertilized ovules of poppy for her Ph.D. work. Scores of media had been tried to induce parthenogenesis but to no avail. In 1964, I had the good fortune of having an enterprising postdoctoral colleague, Dr. Sipra Guha (now Guha-Mukherjee), now herself a Professor in the Jawaharlal Nehru University, New Delhi. She had done some tissue culture work on onion for her Ph.D. under the supervision of Professor Johri, but wanted to do some new physiological and biochemical work with me as a postdoctoral fellow. Of the two research problems I assigned, one was physiological such that her previous training could be utilized. I thus asked her to study the physiology of meiosis in anthers by the tissue culture technique—the idea was to grow anthers or isolated sporogenous cells in culture so that we could manipulate and alter the physical and chemical environment. Many plants, then flowering in our botanical garden, came handy as potential research material. But it was a most unexpected and unbelievable spectacle when one day she brought cultures in cytokinin containing media, containing anthers of *Datura innoxia*, full of embryos. That is how the discovery of mass production method of haploids came about and which has so fascinated botanists all over the world. The anther culture technique of raising haploids has since become common not only in many journals and texts in the areas of tissue culture or plant physiology, but also biochemistry. It is interesting for me to recall that when the discovery came in 1964, everybody was reluctant to believe that the embryos were haploids—I, too, had thought that embryos may have arisen from the anther wall ! Even after we published our second paper in Nature, having meanwhile determined the chromosome numbers in the young plantlets, considerable skepticism remained amongst my colleagues at Delhi. And not until nearly the beginning of the seventies, when Japanese French, and other workers, had fully repeated our experiments and extended the discovery in newer taxa, some trust came in the work ! Today, the literature is so extensive (I hope again the audience will not be disturbed by my admission) that it is hard to remain up-to-date even in a field which we have initiated. The latest count of taxa where production of

haploids has been induced by the anther or pollen culture technique is as follows : 216 species, belonging to 78 genera and 31 families. On the applied front, the major interest is in developing pure homozygous lines and obtaining mutant lines for recessive genes (haploids are useful also for research on fusion of protoplasts since without them, the chromosome number will adversely increase). On the other hand, on the fundamental side, the hope is that new mutants will provide material for research on biochemical genetics of higher plants and thus unfold the mystery of such phenomena as regulation of gene activity in eukaryotes. Methods are now available for the mass culture and production of haploids. An astounding amount of work has been done on crops in China and elsewhere. However, we have restricted ourselves to our good old *Datura* and our own investigations have had a very basic flair. This is partly for the reason that our familiarity with cereal crops or economic plants is not much, and in any case it is not easy to compete with a country like China where whole institutes have been set up for such work (with teams of hundreds). Essentially, we have focussed our attention on how to improve frequency of haploid formation because in many plants haploids cannot be obtained easily. Several physical and chemical treatments have been found useful. Among the former, cold treatment, serial culture (i.e., transfer to fresh medium), and density gradient centrifugation to remove non-embryogenic pollen, are the most influential. In the latter, the removal of inhibitors by charcoal and polyvinylpyrrolidone, and the ability of various chemical regulators like cytokinins, Fe-EDDHA, and glutathione, to stimulate nuclear or cell-division, is worthy of special note. Some recent work in our laboratory shows that the animal neurohormone, acetylcholine, too, may have a strong effect on androgenesis.

At this point, a reference can be made to the new pollen culture technique. Culture of isolated pollen grains can be regarded as a more advanced and sophisticated technique since microbiological procedures like quantitation and replica-plating can be much better applied to pollen than to whole anthers. Unfortunately, pollen culture has so far been very frustrating. Only if one precultures anthers and then isolated pollen or adopts the serial culture method, some success is obtained. Nevertheless, my colleague Dr. Rashid has recently made some spectacular progress. Employing the centrifugation technique with Percoll gradients, he has separated embryogenic and non-embryogenic pollen in *Nicotiana tabacum*, and by this method he has obtained the remarkable value of as many as 40% pollen undergoing androgenesis in the embryogenic fraction.

Some New Areas—The Future

This much about lines of work struck in the mid-sixties and followed up through the seventies. But, finally, I should like to devote the last part to touch on some current studies and some new aspects of my research. Probably, it is correct to acknowledge some debt to a Homi Bhabha Fellowship, to Harvard, and finally to Professor L. Bogorad whose hospitality provided me an opportunity to rethink and record priorities, away from the hustle and bustle of Delhi, as to what should be done on return. (Simultaneously I found some time to work on the mitochondrial RNA polymerase of maize and to familiarize myself with new developments in the nucleic acid field).

To be precise, I should say that investigations on these new aspects (discussed below) were already initiated some years ago, but have been disturbed due to certain circumstances and to an illness that followed. All I hope is that I can be back soon on these researches and follow them more actively.

One of my major efforts has been to reorient cell and tissue culture studies along new lines like mutations and genetic manipulation. Some of you may have read about our recent work on NaCl-resistant cell lines in *Datura*. Another has been on genetic manipulation through virus vectors or nucleic acids. Exciting reports had appeared some years ago from Australia that employing haploids from tomato cells (obtained by the anther culture technique), and λ phage as a carrier the well-known *lac* operon of *E. coli* had been transferred to higher plant cells enabling the callus cells to grow on lactose, a sugar which is normally absent in plants. Similar claims were later made in England in another system. Employing the same technique we have examined whether similar transfer of foreign genetic material is possible in cultures of haploid cell lines of *Datura innoxia*. Unfortunately, our work has failed to confirm transgenosis even though we adopted the same techniques as employed by Australian workers. The paradox is that even though the usual colorimetric technique does indicate enhanced β-galactosidase activity in phage-treated calli, the latter refuse to grow on lactose which is the authentic substrate for this enzyme. It has been a normal practice so far in all work (including that on bacteria) to use ONPG, i.e. p-orthonitrophenylgalactoside, which is an artificial substrate for the assay of this enzyme. We have obviously discovered a possible flaw in the earlier experiments i.e. the β-galactosidase activity may not be genuine since extracts which hydrolyze ONPG are unable to hydrolyze authentic lactose when tested by the radioactive tracer technique. Nonetheless, the moral of these experiments is not that genetic engineering is impractical in plant cells (for which in fact they are very well suited an account of ease of regeneration), but that this work has to be approached along more sophisticated lines with vectors that may be really intgerated in genomes of plants cells like the cauliflower mosaic DNA virus or through plasmids of *Agrobacterium tumefaciens*. But, some beginning has been made, some experience gained in handling viruses and bacteria, and hopefully we should be back at it again if sufficient funds could be had to embark on the recombinant DNA technology which has come up in the meanwhile.

Another aspect, we have tried to develop is the technology of protoplast culture. Obviously, protoplasts are a better material for genetic manipulation than are whole cells. Further, protoplast of one plant can be fused with the protoplast of another, providing new genetic recombinants. The technique is not new of course, but my contribution is to introduce it in the Botany Department of the University of Delhi and in this endeavour we have successfully handled not only a couple of new tobacco species, namely *Nicotiana rustica* and *N. plumbaginifolia*, but some new plants and genera, e.g. *Solanum*, *Capsicum*, *Cyamopsis* and *Vigna* of the Leguminoseae.

Another broad area which I have tried to pursue in the last five years is photobiology, but again work has been interrupted and I hope we can resume it soon. One new sub-project under photobiology has been the photomodulation of enzymes. The rationale for such work is simple.

Processes like flowering although quite spectacular, are very hard to analyze at the molecular level. But clearly, before any visible morphological change, there should be changes in enzyme activity and protein spectrum. With this hope we have been looking into the regulation of the well-known enzyme nitrate reductase in *Lemna*, maize and rice. It turns out that the enzyme in duckweeds and in certain cereals is under phytochrome control, since fairly short exposures to red light would induce enzyme activity. Various experiments have shown that the enzyme is synthesised *de novo*. However, this is as far as we have gone which is not very much. The essersial question is now : how do changes in phytochrome level trigger the synthesis of the enzyme? Quite likely there should be a second messenger, may be calcium or cAMP. If it is possible to have the right opportunity—in terms of facilities—this should be one of the problems to push further.

Another sub-project which touches on phytochrome action is the role of the enzyme acetylcholinesterase. This enzyme until recently was an 'animal' enzyme known to most people as occurring only ia nerves. But because exposure to red and far-red lights can cause instantaneous changes in surface potentials of plant tissues, it was suggested that the same enzyme as occurring in nerves may be found in plants too. But to say the very least the idea is extremely controversial, because people have long ceased to think that a nervous system may exist in plants. We have provided new evidence for he fact that an enzyme very smiilar to that in animals is present in roots of gram and certain other plants. The enzyme has been considerably purified—at any rate, to a better stage of purification than in any other plant system so far. Obviously, if we could pursue this line of work, we would like to find (1) whether ACh-esterase represents an elementary cell-to-cell communication system in plant cells, (2) whether the ACh-esterase system controls the release of Ca^{++} from cell membranes, which in turn could serve as a messenger for phytochrome-mediated response.

A third sub-project is on the intracellular location of phytochrome, since the location is vital for developing further ideas of mechanism of phytochrome action. At least three laboratories have been very active in this field in the world. It has been shown that red light exposure of the tissue binds the Pfr to the membrane so that if one homogenizes the tissue and centrifuges the extract, phytochrome is found in the pellet fraction. Work in our laboratory had easily confirmed this in maize and wheat and cauliflower. However, we seem to have gone a step ahead in the sense that nearly in all work done, so far, modulation of phytochrome binding can be shown, provided the light exposures are given to tissue *in vivo*, i.e. before they are put in the waring blender. However, our unpublished experiments indicate that the binding of phytochrome to membranes by red light can also be achieved, at least in cauliflower, *in vitro*, i.e. even if the exposures are given to extracts and not intact tissue and cells.

CONCLUSION

My concluding remarks are in a bit of a philosophic vein. The question can be asked as to how far we have gone in molecular botany. I am afraid, I have to say, that we are not in it yet but merely approaching it. There are many difficulties, but I hope we can reach our goal given financial support and continuity of work. Of course, financial support

is essential because there are heavy demands on resources in terms of chemicals and new facilities (there are in the audience senior botanists and administrators, including those who have served as Vice-Chancellors or advisors to the Government of India here and I hope they will do everything possible). However, a point I wish to make from my experience is that sometimes our difficulties are as much a matter of funds as that of attitude and even sometimes resistance to the introduction of the physico-chemical approach to studying botany. According to one view, molecular studies are for biochemistry of molecular biology departments which I think is unfortunate. Since I have been misunderstood sometimes, let me make use of this opportunity to make it clear that I am not against the older established disicplines of botany. Undoubtedly, there should be good work in taxonomy, in plant exploration, in ecology, on preservation of flora and forests (such as of the Silent Valley), all of which are absolutely vital for national interest. I am quite positive that even morphology and paleobotany must continne as important disciplines for their intellectual value. As I said at the beginning, who cannot fail to be impressed by concepts such as of Gondwanaland and theory of continental drift ! But if I understand the minds of men like Birbal Sahni or another contemporary of his, my late father, the basic ingredient in their making was not dogged adherence to any sub-discipline but curiosity and the urge for new discoveries, new horizons, truth, and more truth. Even though, for example, advancing years and age were no bar in taking deep interest in physiology and genetics.

I might add that despite the fact that almost all Indian universities have fairly large and long-established Departments of Botany with great potentialities, there are too few centres of research in the new areas. We are thus hardly participating in work such as genetic manipulation, or regulation of gene activity, or the discovery of new hormones or new photoreceptors controlling plant growth and development. My appeal is that we need to reorient ourselves to some extent to introduce new lines of work, new instrumentation to enable use of the physico-chemical approach, and even modify syllabi (after all if new approaches are to be used we need also to prepare the minds of the young in the theory and practice of these approaches). And I do maintain that in pruning a bit from the existing courses and introducing some new aspects, we do not destroy botany but lay the foundations of an even stronger new botany. The sum and substance of what I am saying is that surely the established fields must not only survive and be consolidated, but even as we do so we must accommodate and nurture new areas and I hope that in saying this I carry the message of all the three illustrious men in whose honour we organise these functions.