ADVANCES IN PLANT TISSUE CULTURE AND BIOTECHNOLOGY RESEARCH AT BARC

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Abstract

Plant tissue culture has several applications in areas such as clonal propagation, crop improvement, molecular farming, transgenic research, and production and isolation of bioactive compounds. BARC has been engaged in varied applications of plant tissue culture research since the 1960s and has contributed significantly to micropropagation, crop improvements, process development and understanding of radiation effects on plant cells and transgenic plants. This has significantly contributed to basic and applied research. This chapter summarizes some of the key achievements of plant tissue culture and plant biotechnology since its inception at BARC, Trombay campus. This chapter includes a snapshot of the recent activities and contributions from the group to acquaint a larger platform of interested research scholars, farmers and the industrial fraternity.

1. Introduction

Somatic cells of plants have the same genetic makeup as that of a fertilized zygote, and it was postulated that all plant cells are totipotent. Since the initial studies of plant cells cultured in the test tube by Haberlandt in 1902 and the discovery of plant hormones like auxins and cytokinins, the field of plant tissue culture has blossomed with successful propagation of plant cells *in vitro* through organogenesis or somatic embryogenesis. Rapid developments in cell and tissue culture and genetic engineering have generated

great interest for plant biologists. As our understanding evolved over the decades, plant tissue cultures have found diverse applications in several fields of plant biology, including:

- Micro propagation for the production of a large number of clonal plants.
- Shoot tip culture for virus elimination.
- Development of somaclonal variants.
- Anther or microspore culture for production of haploids.
- Protoplast culture for somatic cell hybridization, cybridization and gene transfer.
- Development of genetically modified plants through *Agrobacterium*-mediated and direct gene transfer methods.
- Production of secondary metabolites in medicinal plant cultures and manipulation of cultural parameters to enhance secondary metabolites as well as manipulation of metabolic pathways.
- Use of plant cells for precise genome editing and crop improvement.

2. Plant Tissue Culture Work at Bhabha Atomic Research Centre

Developments in cell and tissue culture have generated great interest among plant biologists. Plant tissue culture (PTC) emerged as a tool for a number of basic and applied research problems of relevance to plant biology and crop improvement. The plant tissue culture laboratory in BARC was initiated in 1966 by Dr. S. Narayanaswamy who had vast experience in the Department of Botany, Delhi University. Mr E. W. Rajasekhar joined him and helped him in setting up the lab, which needed aseptic conditions for the culture of plant cells. Mr. Rajasekhar went to work in the UK with Professor H. E. Street, who was a doyen of plant tissue culture. The objectives of the plant tissue laboratory in BARC in the initial years were to study the effect of ionizing radiation on plants, morphogenesis and develop regeneration system in various plants.

2.1. Ionizing radiation and plants

Studies concerning the effect of ionizing radiations on seeds, seedlings and callus cultures of *Petunia inflata*, *Antirrhinum majus* and *Pharbitis nil* were initially done at BARC. The striking differences in growth and development of these three types of tissues on exposure to gamma rays were attributed to be organizational differences. One striking observation noted was the stimulation of seed germination in all three plant species at low doses of radiation. The effects of gamma and ultra violet radiations on the survival and totipotency of haploid tobacco cells was also studied.

2.2. Haploid culture, protoplast culture & inter-kingdom hybridization

In 1966, Dr. Sipra Guha and Dr. S. C. Maheswari from Delhi University reported the development of haploid plants from anther culture for the first-time in *Datura innoxia*. Soon after, some pioneering findings were reported on the anther culture in *Datura metel* and in other plants from BARC. As pollen culture was expected to produce mainly the haploids, there were instances of triploid plants instead of haploid plants. The origin of triploids from haploid pollen grains was a mystery and it was postulated that triploids

arose due to the fusion of one generative and two vegetative nuclei in the pollen grains. Later, this was confirmed by Dr. Sutherland from the UK.

Protoplast isolation and regeneration was one of the most exciting research subjects in plant biology worldwide during the 70s. This inspired researchers to undertake studies on plant protoplasts and somatic cell hybridization. The work on *Datura metel* pollen protoplasts was featured in the prestigious journal "Nature" on the cover page (Nature 246, 223-224; 1973). The first successful inter kingdom hybrid between *Amoeba* and *Atropa belladonna* protoplasts using polyethylene glycol and microsurgery was developed. Although there was no fusion of animal and plant nuclei, the plant nuclei divided inside *Amoeba* cytoplasm, which was confirmed by autoradiography. The plant nuclei disintegrated after one month of culture. This is the first report on successful inter kingdom hybridization between an animal and a plant cell (Cytologia 45: 149-155; 1980).

Successful protocols for protoplast isolation and regeneration from *Santalum album* (first for a forest tree), *Nicotiana tabacum*, *Brassica juncea*, *Tylophora indica*, *Pergularia pallida*, *Arachis hypogaea*, *Sesamum indicum*, *Vigna aconitifolia*, *Vigna mungo* and *Catharanthus roseus* were established and plants regenerated in some cases.

2.3. Plant regeneration and genetic improvement in various crops

Extensive research strides took place in the plant regeneration studies in different (and recalcitrant) plant systems, leading to the development of efficient regeneration systems in several economically important crops by organogenesis and somatic embryogenesis.

2.3.1. Cereals, oil seeds and pulses

Untill the 1980s, cereals like wheat were postulated to be recalcitrant, and the regeneration of plants was difficult. Dr. Ingo Potrykus and his group in Switzerland, even after testing hundreds of media combinations, could not get plant regeneration in cereals. For the first time successful plant regeneration in bread wheat using immature embryos and immature inflorescence cultures was reported from BARC. This was reported by the Press Trust of India in the major National newspapers, and there were discussions in Parliament on plant regeneration in wheat. Later, plant regeneration was reported in major cereals and millets like Rye, Triticale, Durum wheat, Sorghum, Bajra, Ragi etc. and oil seed crops like peanut, soybean and mustard. For the first time, a yellow-seeded somaclonal variant was reported in *Brassica juncea*, which was a recessive mutant developed from the black seeded *B. juncea*. Grain legumes are very difficult to regenerate and were thought to be recalcitrant. Regeneration and development of complete plants were demonstrated in several pulse crops such as chickpea, moong bean, black gram and moth bean.

2.3.2. Horticultural crops

In India, BARC was one of the few institutes to take initiatives in the early 1980s to develop a micropropagation protocol for economically important horticultural crops. Soon, efficient regeneration and complete technological protocols in *Santalum album* (sandalwood), *Morus indica* (sericulture), banana and pineapple were developed. Somatic

embryos are a valuable tool for the establishment of long-lasting regenerable tissue, with varied applications in the genetic improvement of plants. For the first time, somatic embryogenesis from male floral buds was reported in banana. A novel approach of somatic embryos production in two bioreactors was developed at BARC. Research on the generation of superior variants of bananas through the development of somaclonal variants and *in vitro* mutagenesis was carried out. Radiation induced mutations in banana cultivar "Giant Cavendish" resulted in the development of a couple of dwarf mutants which are now in advanced trials in collaboration with National Research Centre for Banana, Trichy (**Fig. 1**). One of the them, named TBM-9 is expected to be notified as a new variety very soon. Similarly, a few promising mutants in pineapple with a small crown, large fruit and high sugar content have been screened, and their detailed assessment is underway.

Fig. 1: Dwarf mutant (TBM9) developed in banana. Wild-type cavendish banana (left) displaying lodging due to bunch weight. Bunch development in dwarf mutant (right) of giant Cavendish

Turmeric and ginger are medicinally important spice crops in India. Callogenesis and direct organogenesis from sprouted shoot buds in both of these crops are standardized. Further, *in vitro* mutagenesis is being carried out to create genetic variability for three specific traits, including enhanced rhizome yield, curcumin/ginegerol content, and disease resistance. Field trials of mutagenized turmeric population resulted in a few potential turmeric lines with a higher rhizome yield (up to 1.5-fold higher than control) and curcumin content (4.5-6.5% against 4.2% in control). Similarly, a few promising mutants for resistance/tolerance against rhizome rot-a major disease of turmeric, have been developed.

2.3.3. Ornamental crops: a new opportunity

There is always demand and necessity of new ornamental crop varieties in the modern and rapidly growing floriculture industry. Genetic variations using induced mutagenesis in such crops are introduced for selected traits, including flower morphology, flower colour, compact growth, variegated leaves and disease resistance. A good number of mutants with desired traits are identified in Chrysanthemum (**Fig. 2**), Gladiolus and Carnation, and field trials of the selected mutant lines are going on to develop them as novel varieties. One of the constraints in commercial cultivation of gladiolus is non-availability of a large quantity of propagules. To overcome these limitations, an efficient protocol for the *in vitro* production/multiplication of cormels in gladiolus was standardized.

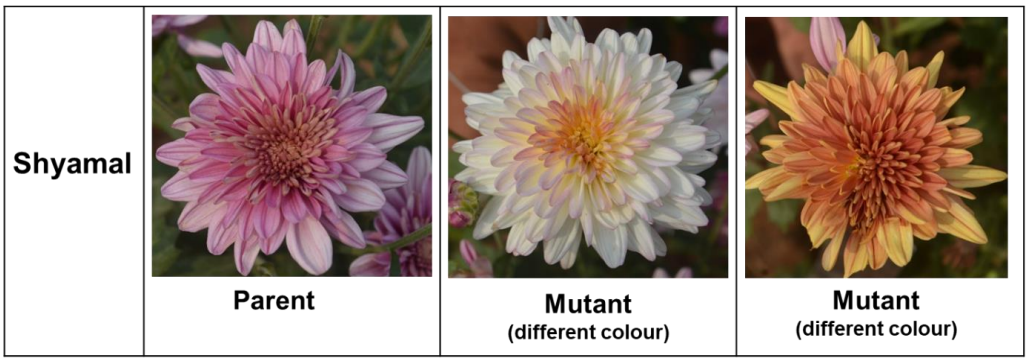

Fig 2: A few promising mutants screened in Chrysanthemum

2.4. Sugarcane

A research program on sugarcane started in the early 2000s, with the idea to employ plant cells cultured *in vitro* for the selection of useful induced mutations at the cellular level. BARC developed a simple and direct method of somatic embryogenesis in sugarcane using segments of immature "inflorescence" of the sugarcane plant. The method was granted an Indian patent (2004, Patent No. 243373). In sugarcane, partial desiccation treatment proved to be beneficial for augmenting growth and plant regeneration in high-dose gamma-irradiated embryogenic callus cultures (**Fig. 3**). This simple and novel

approach can be useful in stimulating the regeneration response of higher dose gamma-irradiated cultures in plants. Studies on *in vitro* mutagenesis using gamma irradiation and *in vitro* selection led to the isolation of several useful mutants for salinity tolerance in popular sugarcane cultivars. Field assessment of a few of them is underway.

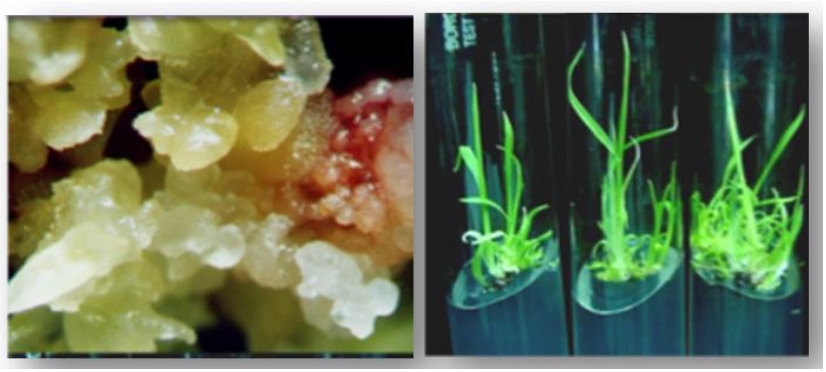

Fig. 3: *In vitro* embryogenesis and plant regeneration in sugarcane

2.5. Synthetic seeds

Somatic embryos and multiple shoots are excellent tools to mass propagate the clonal progeny of vegetatively propagated crops, but they are sensitive and difficult to transport. To overcome it, the concept of synthetic seeds was explored worldwide. BARC demonstrated synthetic seed production and regeneration in mulberry, banana, sandalwood and cardamom.

2.6. Plant secondary metabolites

Medicinal plants constitute an important source for the production of a variety of novel compounds used in therapy. Plant tissue cultures initiated from these medicinal plants offer an excellent source for the production of these important secondary metabolites without the destruction of the entire tree/natural habitat. Research aimed to explore secondary metabolites in tissue cultures of various medicinal plants, like *Tylophora indica, Atropa belladonna, Physalis minima, Catharanthus roseus, Rauwolfia serpentina, Holarrhena antidysenterica, Tinospora cordifolia, Azadirachta indica, Artemisia annua, Psoralea corylifolia, Andrographis paniculate, Taxus baccata* and *Coleus* sp. A bioreactor facility for the plant cells was established and the stepwise procedure of scale up of cultures from flasks to bioreactors was consistently demonstrated.

Work on haploid *Atropa belladonna* cultures showed that tropane alkaloids could be synthesized under tissue culture conditions, contrary to the earlier reports. *Taxus baccata* callus cultures revealed significant levels of 10-deacetyl baccatin III-a precursor for the semi-synthesis of taxotere, a novel anticancer agent with improved solubility than taxol. A two-stage method proved effective for obtaining good growth and production of taxanes. Organized shoots, suspension culture and hairy roots induced by *A. rhizogenes*

in a few medicinal plants were scaled-up in bioreactors and their alkaloid synthesis studied. Realising the importance of Podophyllotoxins and their derivatives in cancer therapy, attempts were made to develop tissue culture methods for the production of these lignans. An alternate plant species namely *Linum flavum*, widely occurring in the European countries, was found to be an ideal source for the lignan 5-methoxypodophyllotoxin (5-MPT). Genetic transformation studies using *Agrobacterium rhizogenes* enhanced the levels of 5-MPT significantly. Later, work focused on *Nothapodytes foetida* and *Ophiorrhiza* sp.- the two endangered Indian medicinal plants and source of prominent anticancer compound- camptothecin (CPT). For enhancing the production of bioactive molecules in the target plants, elicitation, immobilization, and the development of hairy roots cultures are also being carried out regularly. Induced mutagenesis using various mutagens can be one of the effective tools for enhancing secondary metabolites production in medicinal plants. A significant enhancement of CPT production in gamma-radiation treated *Nothapodytes foetida*'s callus culture over control was reported. To enrich the understanding of camptothecin biosynthesis in *Ophiorrhiza rugosa*, tissue-specific "OMICS' based analyses were undertaken. Together with various functional validation strategies, several crucial players of CPT biosynthesis could be identified, and are being used for targeted metabolic engineering of terpenoid indole alkaloid (TIA) biosynthesis.

2.7. Pioneering work in plant transgenic research

Transgenics are valuable tools for trait introduction and for basic research on gene characterization. Consequent to the first report in 1983 on the genetic transformation of tobacco using *Agrobacterium* by Dr. Jeff Schell and his team from Germany, experiments were initiated at BARC for the development of genetically modified plants. Soon transgenic moth bean plants with a bacterial gene (*npt*II) were developed using three different techniques, such as *Agrobacterium*-mediated, PEG-mediated, and electroporation. This was the first report on development of a transgenic pulse crop from protoplasts. Later, c*ry1Ac* gene from *Bacillus thuringiensis* was introduced into chickpea and moth bean by *Agrobacterium* and direct gene transfer using particle gun and transgenic plants with improved tolerance to insect pests were developed. BARC developed transgenic groundnut plants with a foreign gene for the first time. Introduction of a synthetic *cry 1Ac* gene in Indian mustard improved tolerance to insect pests, while the introduction of a chitinase gene from fungus *Trichoderma virens* into tomato enhanced fungal resistance. Similarly, over-expression of an anti-microbial peptide gene (MSI–99, magainin) in banana and tobacco improved disease resistance in both the plants. Work on coat protein genes for disease resistance has been done on Potato and transgenic potato plants were raised and tested at Potato Research Laboratory. Shimla,

Molecular farming is an attractive option for vaccine production using plants system. In this direction, attempts were made and BARC published first report on the genetic transformation of banana cv. Rasthali, an elite Indian variety of banana. At BARC, hepatitis B surface antigen (HBsAg) was expressed in tobacco, tomato, soybean, banana and potato, and its expression was demonstrated in fruits of banana and tomato and

microtubers of potato. These studies encouraged many research institutes in their efforts for development of edible vaccines. The banana transformation system has been followed extensively for the development of transgenic banana plants and on understanding the gene functions in biotic and abiotic stress tolerance, secondary wall deposition, xylem development, biofortification and shoot multiplication. Work on understanding the nature and activities of the native promoters of banana has helped to decipher their basic mechanisms. Fortified banana developed by overexpressing *nicotianamine synthase 2* (*OsNAS2*) gene of rice in banana (an India-Australia technology transfer project) showed higher accumulation of Fe (17 times) and Zn (12 times) in the selected transgenic lines compared to control. Contained advance field trials of the promising transgenic banana lines are being carried out at National Research Centre for Banana (NRCB), Trichy.

Transgenic research as a tool has also been demonstrated in the field of phytoremediation. To enhance the potential of plants to degrade organic pollutants, two genes namely a human cytochrome P450 and a fungal glutathione S-transferase, were introduced into tobacco, and transgenic plants were shown enhanced degradation of radiolabelled pesticides. Introduction of a fungal Zn transporter and a fungal Cu transporter into the plants led to enhanced removal of Zn and Cu, respectively from soil and solution. Further, plants expressing a Ni-Co transporter gene could take up high levels of radioactive cobalt compared to control plants. However, these transgenic plants could not be evaluated under the field conditions due to restriction on field testing of genetically modified plants, but efforts are going on to translate these findings.

3. Spinoff technologies

Developing efficient micropropagation system is a prerequisite to achieve significant progress in *in vitro* mutagenesis. At BARC, micropropagation technologies for some of the important crops were developed for their varietal improvement program. Tissue culture technologies for the vegetative propagation of commercially important horticultural crops such as banana (AB10NABTD**)**, pineapple (AB44NABTD), ginger (AB49NABTD), turmeric (AB40NABTD), and the medicinal plant *Ophiorrhiza rugosa* (AB01NABTD) were developed and transferred to several end-users. These methods have several advantages over traditional propagation approaches, including efficient generation of a large number of clonal and disease-free plantlets that can be disseminated to farmers for cultivation.

4. International/National collaborations

BARC had an Indo-FRG bilateral program during the 1980s and many BARC Scientists visited laboratories in Germany. Scientists from abroad (Russia, Cuba and Syria) were trained in the lab. The plant tissue culture lab was involved in an Oil Palm Multiplication project by Department of Biotechnology, Govt. of India (GOI) and a Cotton Mini Mission project of GOI. Under a DBT-BIRAC Indo-Australian Biotechnology project, the genetic constructs received from Queensland University of Technology, Australia,

were transferred in Indian banana cultivars for iron bio-fortification. Promising banana lines with significantly elevated iron content are being tested under controlled field trials at National Research Centre for Banana (NRCB), Trichy. Scientists with expertise in plant tissue culture participated in expert missions on *in vitro* mutagenesis in crop plants, and in several International Atomic Energy Agency (IAEA, Vienna) sponsored Coordinated Research Projects on radiation induced mutations including, an ongoing project on potato.

Collaborative work with other national research organizations and universities was a major ongoing activity, and accordingly several MoUs were signed. Productive research outcome was achieved from these collaborations. Students and faculties from all over India were trained in plant biotechnology on a regular basis.

5. Precise genome editing in plants: the way forward

Precise genome editing is the way forward for the genetic improvement of crops. As per the recent DBT guidelines, genetically modified plants, free of transgene insertion with limited editing of bases, do not fall under preview of regulations for transgenics, and can be released as mutants. With an experience of several decades in efficient regeneration and transformation systems in different crops, BARC took early initiatives on the entablement of protocols for gene editing of crops using tissue culture and CRISPR-Cas9 based systems. Recently, researchers at BARC reported editing of SNAC67 gene, a master regulator of stress-induced senescence, and its roles in salicylic acid dependent senescence induction in banana plants. Further, silencing lipoxygenase genes led to an improvement in resistance of banana plants towards *Fusarium oxysporum*. Similar efforts are going on in other important crops.

6. Acknowledgements

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