# GENETIC IMPROVEMENT OF MEDICINAL AND VEGETATIVELY PROPAGATED CROPS THROUGH INDUCED MUTAGENESIS: ROLE OF PLANT TISSUE CULTURE TECHNOLOGY

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**Abstract:**

Genetic diversity and selection in nature have been primary sources of any crop improvement breeding program. This diversity results from naturally occurring heritable changes in genetic material, commonly called 'mutations'. But such mutations happen at a very slow rate. Alternatively, induced mutagenesis in which explants can be treated with a mutagen, can accelerate the rate of random mutagenesis, resulting in desired mutations economically. This chapter summarises the progress made in the genetic improvement of selected medicinal and vegetatively propagated crops at our institute using induced mutagenesis in combination with modern tools of plant tissue culture technology.

**Keywords:**

Micropropagation, somaclonal variation, *in vitro* mutagenesis, plant secondary metabolites, spin-off technologies.

## 1. Introduction:

A wide range of crops are not propagated through seeds, but being multiplied using vegetative plant parts i.e. stem, root, tuber, leaves and cuttings etc. Such crops are known as vegetatively propagated crops (VPCs) and include several agronomically important crops such as banana, pineapple, potato, ornamentals, sugarcane etc. Vegetatively propagated progenies are always a clone of their parent plant and cannot acquire genetic variability through its propagation. Due to their limited genetic variability, vegetatively propagated crops are prone to diseases arising due to continuous evolution of pathogens. Conventional plant breeding depends on the availability of genetic variation for crossing and selection of desired genotypes. However, most VPCs are not suitable for such methods. Here plant tissue culture technology, and induced mutagenesis, in combination with modern tools of plant biotechnology offers an excellent alternative approach in producing novel genotypes in a crop of our interest.

Plant tissue culture is a technique where cell or tissue of plants commonly referred as explants are grown *in vitro* which literally means growth under aseptic and controlled conditions. Plant tissue culture technologies have gained huge industrial importance in recent years for their applications in crop propagation, genetic improvement, disease eradication and secondary metabolites production[3]. Through plant tissue culture technologies, more commonly called as micropropagation collectively, small explants can give rise to thousands of identical plantlets in a short period, round the year irrespective of the season and weather conditions. It is very useful in rescuing endangered plant species of medicinal or agricultural importance.

The bottleneck of lack of genetic variability in VPCs has been tackled through the efficient use of plant tissue culture technology by generation of 'somaclonal variants'[10]. These are clones that are genetically different from their parent and are generated occasionally during micropropagation, particularly from undifferentiated cellular mass ie callus. These variants are sometimes of superior quality in comparison to their parental counterparts and are widely used in development of improved plant types. However, identification of superior quality somaclonal variants is a tedious procedure due to low frequency in generation of somaclonal variants. Hence, identification of somaclonal variants can sometime solely rely on performance of the crop in terms of growth, productivity or disease resistance but should be supplemented biochemical and molecular confirmations. To combat issues related to the generation of somaclonal variants, *in vitro* mutagenesis is widely used to create genetic variability in vegetatively propagated crops. During *in vitro* mutagenesis, tissues or cells of vegetatively propagated crops are exposed to chemical or physical mutagens and then sub-cultured for six-eight generations for the segregation of novel mutants[22]. A spectrum of random mutations in this process easily generates an array of genetically diverse plants, which can then be conveniently screened using tissue culture techniques for anticipated characteristics[18]. Besides being laborious, other drawbacks of this technique are the random mutagenesis and the requirement of considerable space to culture and screen the mutants, followed by validation of their performance in field conditions. Recent advances in genetic engineering have led us to the development of targeted mutants through CRISPR-Cas9 based genome editing technologies in vegetatively propagated crops.

Nuclear Agriculture and Biotechnology Division (NA&BTD) of BARC has been involved in the plant tissue culture research since its foundation days and has contributed to all the major aspects of plant tissue culture technology and genetic engineering. Some of the pioneering work in the field of anther culture, protoplast culture including interkingdom fusion were done at our lab[16]. The first transgenic plant in the world was reported in 1984, and by 1987, we could develop the first transgenic legume (mothbean) with a bacterial gene[5]. NA & BTD has played a significant role in developing tissue culture technologies for the vegetative propagation of commercially important horticultural crops such as banana, pineapple, ginger, turmeric and the medicinal plant *Ophiorrhiza rugosa*. We have generated novel mutants in banana, pineapple, turmeric and ornamental crops through somaclonal variation and *in vitro* mutagenesis. Recently, we developed techniques for targeted mutagenesis using the cutting edge CRISPR-Cas9 based genome editing and generated novel banana mutants[15]. In this chapter, we will discuss recent advancements, technologies and achievements of the NA&BTD in the field of genetic improvement of medicinal and vegetatively propagated crops using irradiation and/or plant tissue culture technology.

## 2. Genetic improvement of Indian medicinal plants:

Plants provide a seemingly inexhaustible and structurally diverse phytopharmaceuticals of simple and complex structures. Many of them, which are relatively less abundant and synthesized by specialized metabolism in plant belong to a class of compounds called as 'plant secondary metabolites'. The numbers of these metabolites are very large, and their presence in plants, being sessile organisms, give them a competitive advantage in their own environment. They have several applications in the fields of human healthcare, agriculture, food industry etc. A large number (>25%) of the pharmaceutical drugs used are derived either directly or indirectly from plants with an annual sale of these products exceeding 30 billion dollars in the USA alone[6]. Conventionally these compounds are either isolated naturally from target plants or by artificial synthesis. The chemical synthesis of many of these molecules are relatively complicated and economically less of unviable. The increasing demand and limited natural resources of such compounds led to the over-exploitation of the target plants. Therefore, there is a need to find sustainable alternative resources for such high value compounds. Plant tissue culture has proved to be an alternate approach for production of bioactive phytochemicals. At NA&BTD, we have carried out research work on several important medicinal plants of Indian origin such as *Ophiorrhiza rugosa*, *Nothapodytes foetida*, *Catharanthus roseus, Azadirachta indica, Artemisia annua, Psoralea corylifolia* and *Andrographis paniculata*. Medicinally active compounds such as camptothecin (CPT), azadirachtin, phytoestrogens namely daidzein and genistein, andrographolide have been isolated in the lab using various plant tissue culture approaches[7,17]. We have developed cell suspension, multiple shoot and hairy roots cultures of these plants and also scaled-up their production in bioreactors for large scale production of bioactive compounds[12,19]. 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[20]. Induced mutagenesis using various mutagens can be one of the effective tools for enhancing secondary metabolites production in medicinal plants. We have demonstrated for the first time an enhancement of CPT production by 20% over control in gamma-radiation

treated *Nothapodytes foetida*'s callus cultures[8]. Similarly, callus cultures of *Rubia cordifolia* irradiated with gamma radiation showed 6 and 11-fold higher alizarin and purpurin contents, respectively than non-irradiated cultures[2]. Apart from induced mutagenesis in combination with tissue and cell culture methods, knowledge-based metabolic engineering and controlled transgene expression of key gene (s) can help to achieve enhanced production of compound of our interest in a target plant. To enrich our understanding of camptothecin biosynthesis in *Ophiorrhiza rugosa*, we did tissue-specific transcriptome and metabolome profiling. Together with various functional validation strategies, we could identify a few crucial players of CPT biosynthesis, and using them for targeted metabolic engineering of terpenoid indole alkaloid (TIA) biosynthesis. In one of our recent studies, overexpression of strictosidine synthase led to two-fold increase in CPT in *O. rugosa*[21].

## 3. Genetic improvement of vegetatively propagated crops:

Improving vegetatively propagated crops via efficient and economic routes is a challenge and radiation induced *in vitro* mutagenesis can be an approach to overcome this challenge[13]. For such crops, i*n vivo* (cuttings, corms, rhizomes etc) or *in vitro* samples (multiple shoots, callus etc) of these plants are first irradiated with an optimized dose of gamma-rays. Irradiated population after a few rounds of subcultures (to generate chimera-free lines in case of *in vitro* tissues) are screened for desired variations and characters. Once the stability of character (s) is established, the selected lines undergo extensive field trials of two-three years. Based on their superior performance against controls/checks, the selected lines can be identified for release as a new variety (ies).

### 3.1. Achievements in fruit crops:

#### 3.1.1. Banana:

Banana is a nutritionally rich wholesome fruit and globally is an agriculturally important fruit crop. We have been working on the generation of superior variants of bananas through the development of somaclonal variants and *in vitro* mutagenesis. We developed a dwarf mutant of a commercially important banana cultivar "Giant Cavendish" (Fig. 1). Giant Cavendish cultivation regularly encounters loss of productivity because of its long height which makes it prone to lodging due to heavy bunch weight during the fruiting season. Hence, a dwarf mutant resistant to lodging in Giant Cavendish was highly desirable and was thus isolated from its multiple shoots exposed to gamma irradiation. This mutant named TBM-9 is currently under the advanced stages of field trials and is expected to be notified by ICAR as a new variety very soon.


**Fig. 1: Dwarf mutant (TBM-9) in banana cv. Giant Cavendish**

Other than conventional approaches of mutant generation for genetic improvement of banana, we also established an efficient *Agrobacterium*-mediated transformation technique for the generation of transgenic banana plants. This transformation system is a valuable tool for understanding gene functions and for introducing novel characteristics in banana plants. We developed iron and zinc fortified transgenic banana in collaboration with Queensland University of Technology, Australia. This was achieved by overexpressing *nicotianamine synthase 2* (*OsNAS2*) gene of rice in banana which led to higher accumulation of Fe (17 times) and Zn (12 times) in the selected transgenic lines compared to control (Unpublished data). Contained advance field trials of the promising transgenic banana lines are being carried out at National Research Centre for Banana (NRCB), Trichy. Recently we generated a few precisely targeted mutants in banana using cutting edge genome editing tool like CRISPR-Cas9[15]. Along with the crop improvement programme the division is also actively involved in basic research on banana. Using transgenic and molecular biology approaches we have characterized many important genes which are involved in secondary wall biosynthesis, disease resistance, abiotic stress tolerance and stress induced senescence[9].

### 3.1.2. Pineapple:

Globally, pineapple is an important fruit crop because of its nutritional value and contribution to the economy of farmers. We are also involved in the pineapple genetic improvement programme through *invitro* mutagenesis. We are working on genetic improvement of two commercially important cultivars of pineapple viz. Queen (spined leaf cultivar) and MD2

(smooth leaf cultivar). The targeted traits for pineapple improvement are fruit size, reduced crown size, disease resistance and early flowering. Multiple shoots of pineapple growing *invitro* were treated with different doses of gamma radiation and through several rounds of subculturing a mutagenized population was generated. During field trial, we screened a few promising mutants in pineapple cultivar Queen with desirable small crown, large fruit and high sugar content characteristics. Further large-scale field trial of the selected mutant lines will be carried to assess their performance and develop them as novel variety if found superior.

### 3.2. Achievements in turmeric:

Turmeric (*Curcuma longa* L.) belonging to the family Zingiberaceae is commercially as well as medicinally important spice crop of India. Curcumin, a bright yellow coloured polyphenolic active constituent present in its rhizomes imparts medicinal properties to this crop. Due to its multipurpose medicinal properties, especially immuno-boosting and strong antioxidant properties, turmeric with high curcumin content is in high demand by pharmaceutical industries. Different *in vitro* culture systems for turmeric, such as callogenesis and direct organogenesis from sprouted shoot buds have been established in our laboratory. We have been engaged in *in vitro* mutagenesis in turmeric for creating genetic variability for three specific traits including enhanced rhizome yield, curcumin content, and disease resistance. For enhanced rhizome yield and curcumin content, promising turmeric lines regenerated from post-irradiated callus and *in vitro* selection, were subjected to field assessment. During field trials, a few potential turmeric lines with higher rhizome yield (up to 1.5-fold higher than control) and curcumin content (4.5-6.5% against 4.2% in control) were identified. Rhizome rot is a major disease of turmeric which causes almost 50% losses of this crop. We have been trying to develop rhizome rot resistance in turmeric using *in vitro* mutagenesis. Promising lines obtained from post-irradiated callus growing on selection medium are being further assessed for disease resistance in green house and field conditions.

### 3.3. Achievements in floriculture/ornamental crops:

There is always demand and necessity of new ornamental crop varieties for modern and rapidly growing floriculture industry. The possibilities for creating different forms and improving ornamentals are infinite, and induced mutagenesis can be an important tool in assisting the same[4]. Induced mutagenesis is a highly effective method for creating genetic variability in ornamental plants with desirable characters expected within a given species' genetic scope[23]. So far, among more than 3400 officially released mutant varieties worldwide, 566 represent ornamental plants (http://www-mvd.iaea.org). Looking at scope and need of the work, genetic improvement of floriculture work was initiated at BARC a few years back. We are working on such crops for the selected traits including flower colour, flower morphology, compact growth, variegated leaves and disease resistance using induced mutagenesis.

### 3.3.1. Chrysanthemum (*Chrysanthemum* sps.) :

Chrysanthemum (locally called 'Shrevanti' or 'Guldaudi') is one of the most popular flowers of India. It is used both as a commercial flower crop as well as a popular exhibition/cut flower. It has earned tremendous popularity in the floriculture industry due to a wide range of flower colour, form, and excellent keeping quality. The unique position of chrysanthemum may be attributed to their wide adaptability to varied agro-ecological conditions. Based on popularity and desired agronomic characters, eight verities of chrysanthemum were selected and their cuttings were irradiated with optimized doses of gamma-radiation. We identified a good number of promising stable chrysanthemum mutants (Fig. 2A) and many of them showed better performance than their parent for selected desired traits.

### 3.3.2. Gladiolus (*Gladiolus grandiflorus* L.) :

Gladiolus, the queen of bulbous flowers, is a member of Iridaceae family. It has high commercial value for its long shelf life, widespread cut flower uses, in making bouquet, flower arrangements and for indoor decorations. As gladiolus is a vegetatively propagated crop, its improvement is difficult using traditional methods. Here, mutation breeding offers a great potential and we are working in this direction. We irradiated corms of commercially important varieties of gladiolus with optimized doses of gamma radiation and irradiated population was screened for the variation. Promising variants in the selected gladiolus varieties were identified and these variants were assessed for stability and other agronomic traits in further generations (Fig. 2B).

Gladiolus faces another challenge of multiplication of corms which is a slow process. It is vegetatively propagated by underground grown corms and cormels, however, one of the constrains in commercial cultivation is non-availability of a large quantity of propagules. To overcome these limitations, we have developed an efficient protocol for the *in vitro* production/multiplication of cormels in gladiolus. This micropropagation system has been tried for three important commercial varieties of gladiolus ie., Psittacinus Hybrid, White prosperity and Joska. Such a method will also help us overcome the limitation of corms availability required for the trials of a selected mutant.

### 3.3.3. Carnation (*Dianthus caryophyllus* L.) :

Carnation is the second most popular and commercially important flower in the world after rose. It is preferred for an excellent keeping quality, a wide range of colours and long shelf-life[1]. Europe and the USA cultivate this crop on large area where as in India it is cultivated on a relatively smaller scale. Himachal Pradesh, West Bengal, Jammu & Kashmir, Uttarakhand, Tamil Nadu and Karnataka are major carnation producing states. There is a huge demand for the newer variants for commercial cultivation and we initiated work on this aspect. Rooted plantlets of carnation (var. BIZET) were irradiated with different doses of gamma rays and three stable mutants showing different flower colour from the parent were isolated (Fig. 2C). The stability of trait in these mutants has been confirmed in the advanced generation (M1V4) and their performance has been found at par with the parent. These mutants will be multiplied and taken forward for detailed field trials.

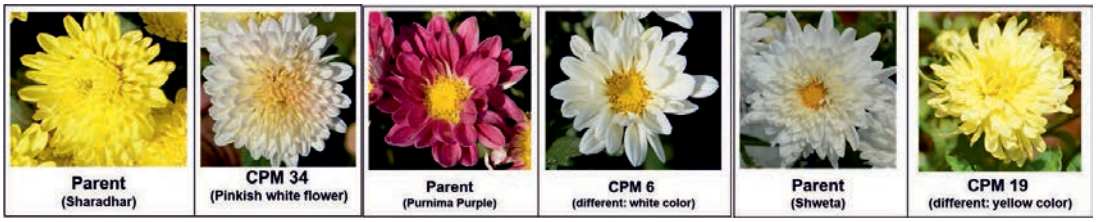


**Fig. 2A: Stable mutants in Chrysanthemum**

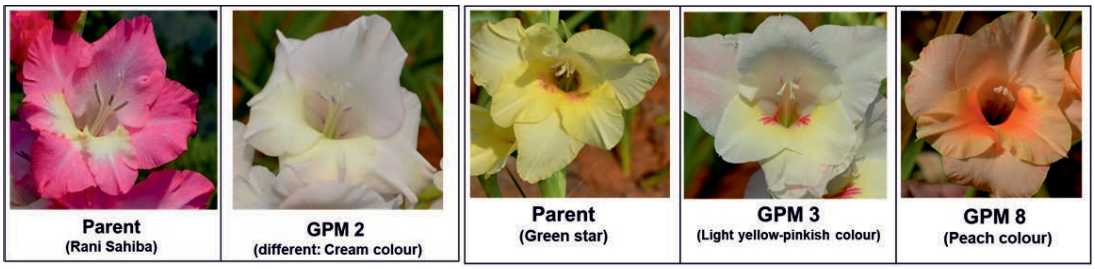


**Fig. 2B: Stable mutants in Gladiolus**

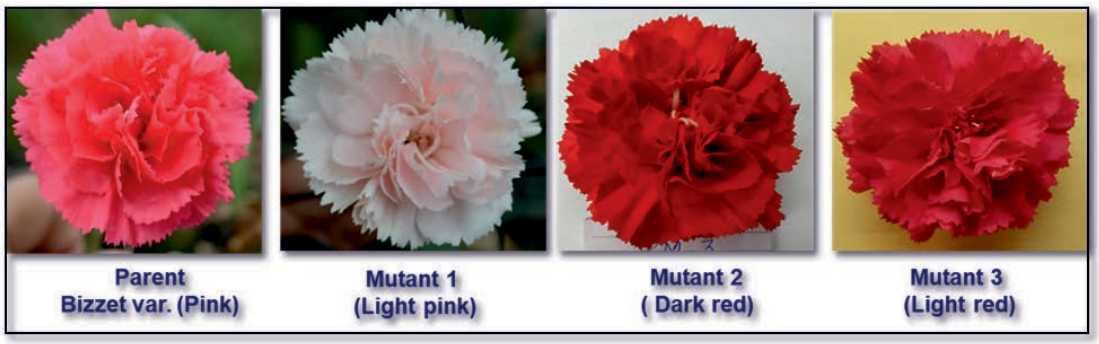


**Fig. 2C: Stable mutants in Carnation**

## 4. Spinoff technologies:

Developing efficient micropropagation system is a prerequisite to achieve significant progress in *in vitro* mutagenesis. We, at BARC developed micropropagation technologies for some of the important VPCs in our journey of the varietal improvement program. These methods have several advantages over traditional micro propagation such as (1) a large number of plantlets can be generated from a single plantlet in a short duration and hence, the problem of planting material shortage can be easily solved; (2) the crop can be cultivated during any period of the year due to rapid availability of healthy plantlets; (3) plantlets developed from tissue culture technology are disease-free and hence can ensues better productivity under uncertain conditions; (4) plantlets originating from such technology are consistent in age and maturity resulting in uniform harvest time in the field. This results in significant reduction in recurrent charges for harvesting and transport of crop to market; (5) this technology has opened up avenues for rapid conservation of biodiversity as exotic

and endangered cultivars can be quickly multiplied and disseminated to farmers for cultivation.

### 4.1. Technology for rapid, continuous and renewable multiplication of *Ophiorrhiza rugosa* as a source of anti-cancer drug Camptothecin (Ref: AB01NABTD):

Camphthecin (CPT) is a monoterpenoid indole alkaloid isolated from plants. It exhibits anti-tumor activity due to its ability to inhibit topoisomerase I[11]. The water-soluble derivatives of camptothecin namely-irinotecan and topotecan have been widely used for treatment of metastatic colorectal cancer, ovarian cancer, small lung cancer, cervical cancer[24]. Due to complex structure, chemical synthesis of CPT is either difficult or not economical. In contrast, plants can easily accumulate these compounds under natural conditions. This has led to the exploitation of numerous medicinal plants for the production of life-saving medications. Plant cell and organ cultures have been recognised as an alternate and renewable source, which can be used for continuous production of secondary metabolites. For camptothecin, BARC has developed a method for micropropagation of *Ophiorrhiza rugosa* that provides a continuous and sustainable source of numerous uniform size plants throughout the year, without threatening the plant`s natural habitat (Fig. 3A). The technology is transferred to six companies/end users including Patanjali Group, Haridwar.


**Fig. 3A: Micropropagation protocol for Ophiorrhiza rugosa**

### 4.2. Large scale micropropagation system in banana (Ref: AB40NABTD):

The majority of cultivated edible banana varieties are seed less and hence are propagated vegetatively. The conventional propagation of edible banana cultivars is through side suckers and generally a single banana plant can generate 5-10 side suckers in 12-18 months. Due to low multiplication potential and long generation period of banana, its production faces shortage of planting material during peak seasons of its cultivation. However, the plant tissue culture technology developed by us offers unlimited supply of banana platelets round the year for the farmers (3B). This technology is very popular among the agricultural companies in India and is transferred to >30 six companies/end users.

### 4.3. Technology for micropropagation of pineapple (*Ananas comosus* L.) (Ref: AB44NABTD):

Pineapple is propagated through slips and side suckers. A pineapple plant generally produces 4-5 suckers in one year limiting the expansion potential of pineapple cultivation. Moreover, the inconsistency in growth of fresh suckers and slips generally leads to nonuniformity in

flowering and can lead to reduction in profits due to incidences of multiple harvests and transports. Generation of planting material for the next generation of crop is time consuming hence, the only way out is multiplication through *in vitro* propagation. We developed a widely accepted tissue culture technology for pineapple micropropagation that offers steady production of uniform pathogen-free plantlets round the year (3C). The technology is transferred to an Agro-company.


**Fig. 3B: Micropropagation protocol for Banana**

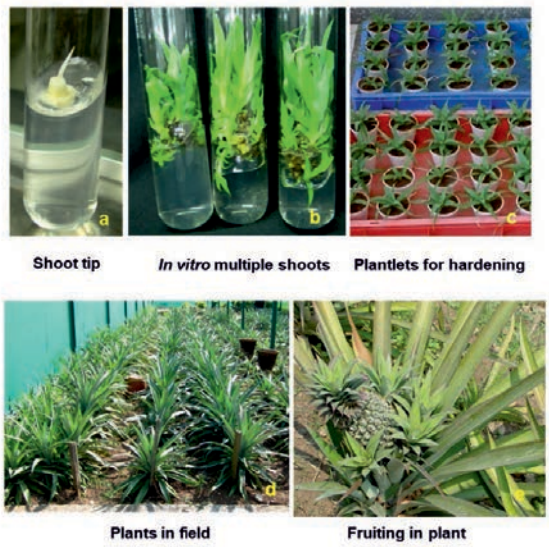


**Fig. 3C: Micropropagation protocol for Pineapple**

### 4.4. A rapid, reproducible and sustainable micropropagation technology for turmeric (*Curcuma longa* L.) (Ref: AB40NABTD):

Being a sterile triploid, turmeric is propagated vegetatively through its underground rhizomes. Slow rate of rhizome multiplication, slow growth of sprouted buds, limited availability of high yielding genotypes, expensive field maintenance of planting material and transmission of diseases from mother rhizomes to developing plants are major constraints in turmeric propagation. Micropropagation of turmeric has always been advantageous over-conventional propagation as it is possible to produce a large number of true to the type, disease-free plants in a relatively short span of time and space. We have developed a rapid and efficient micropropagation protocol for clonal propagation of turmeric. Numerous plantlets of turmeric can be produced from a single explant in a year using this protocol. The plantlets showed simultaneous rooting in the same medium which minimizes labour, cost and time required for 2-3 subcultures of the *in vitro* cultures (Fig. 3D). The healthy disease -free plants obtained through this method ultimately improve the yield potential of this crop. This

micropropagation protocol is also useful for germplasm conservation of turmeric. The technology is transferred to five end users till now.

### 4.5. An efficient, rapid and reproducible micropropagation protocol for ginger (*Zingiber officinale* L.) (Ref: AB49NABTD):

Ginger belongs to the family Zingiberaceae and is one of the world's most important spice crop used in pharmaceutical and culinary purposes[14]. It has been used as a herbal medicine for the treatment of numerous diseases and also been recognized as an immunity booster by Ministry of AYUSH. Ginger is propagated asexually through its rhizomes. With slow proliferation rate, and the reproducing part (rhizome) is also the economically used part of ginger, it restricts the availability of planting material needed for cultivation. Also, rhizomes are easily infected by soil-borne pathogens. Micropropagation, especially for vegetatively propagated crops, is as an alternate tool for fast clonal multiplication of pathogen-free plants. We have developed a mass propagation technology for ginger using sprouted buds of rhizomes. The protocol offers numerous uniform-size and disease-free planting material throughout the year and can enhance potential productivity of this crop (Fig. 3E). This technology can also be used in the conservation of elite cultivars of ginger. The technology is transferred to two end users.

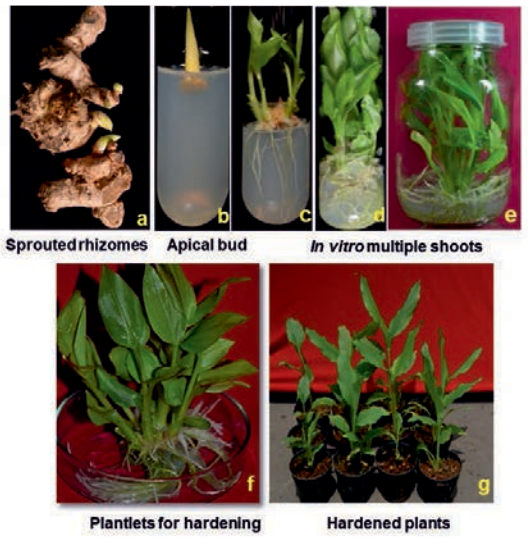


**Fig. 3D: Micropropagation protocol for Turmeric**

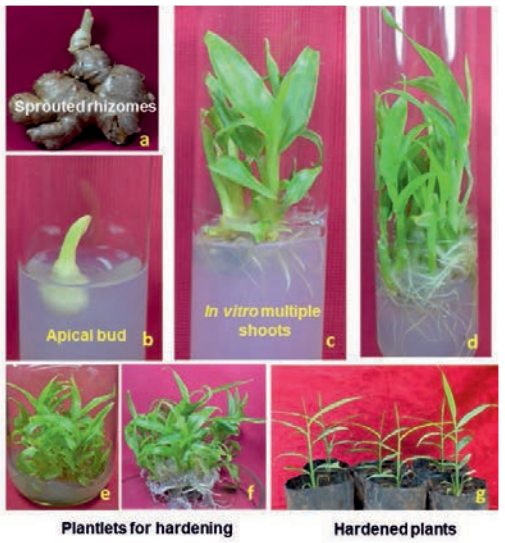


**Fig. 3E: Micropropagation protocol for Ginger**

### 5. Conclusions:

The genetic improvement of vegetatively propagated crops for desired traits is a continuous process. Conventional plant breeding depends on the availability and utilization of genetic variation for crossing followed by selection of desired genotypes from different populations.

However, most VPCs are not suitable for such methods. The possibilities for creating variations are infinite, *in vitro* mutagenesis, aided by plant tissue culture offers an excellent alternative approach in producing novel genotypes in agronomically important vegetatively propagated crops.

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