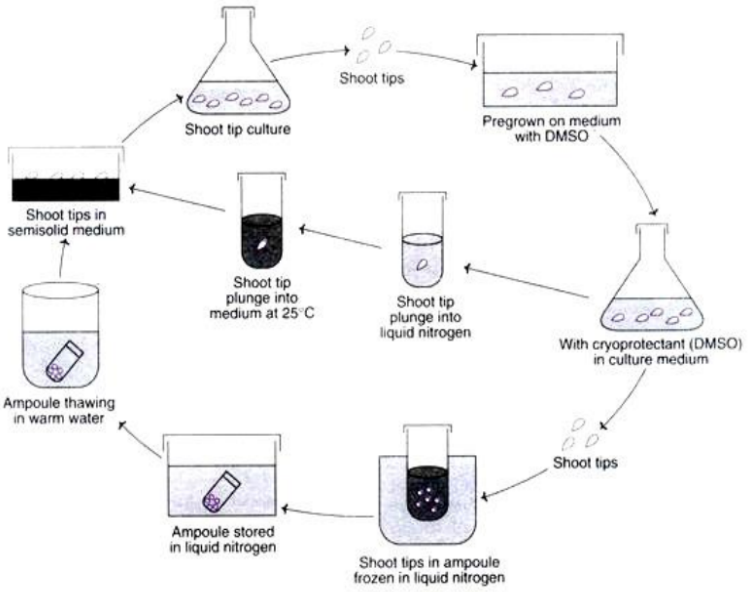
SOMACLONAL VARIATION IN PLANTS CONTINUED

Technique of Cryopreservation:

An outline of the protocol for cryopreservation of shoot tip is depicted in Fig. 1. The cryopreservation of plant cell culture followed by the regeneration of plants broadly involves the following stages



1. Development of sterile tissue cultures
2. Addition of cryoprotectants and pretreatment
3. Freezing
4. Storage
5. Thawing
6. Re-culture
7. Measurement of survival/viability
8. Plant regeneration.

The salient features of the above stages are briefly described.

Development of sterile tissue culture:

The selection of plant species and the tissues with particular reference to the morphological and physiological characters largely influence the ability of the explant to survive in cryopreservation. Any tissue from a plant can be used for cryopreservation e.g. meristems, embryos, endosperms, ovules, seeds, cultured plant cells, protoplasts, calluses. Among these, meristematic cells and suspension cell cultures, in the late lag phase or log phase are most suitable.

Addition of cryoprotectants and pretreatment:

Cryoprotectants are the compounds that can prevent the damage caused to cells by freezing or thawing. The freezing point and super-cooling point of water are reduced by the presence of cryoprotectants. As a result, the ice crystal formation is retarded during the process of cryopreservation.

There are several cryoprotectants which include dimethyl sulfoxide (DMSO), glycerol, ethylene, propylene, sucrose, mannose, glucose, proline and acetamide. Among these, DMSO, sucrose and glycerol are most widely used. Generally, a mixture of cryoprotectants instead of a single one is used for more effective cryopreservation without damage to cells/tissues.

Freezing:

The sensitivity of the cells to low temperature is variable and largely depends on the plant species.

Four different types of freezing methods are used:

1. Slow-freezing method:

The tissue or the requisite plant material is slowly frozen at a slow cooling rates of 0.5-5°C/min from 0°C to -100°C, and then transferred to liquid nitrogen. The advantage of slow-freezing method is that some amount of water flows from the cells to the outside. This promotes extracellular ice formation rather than intracellular freezing. As a result of this, the plant cells are partially dehydrated and survive better. The slow-freezing procedure is successfully used for the cryopreservation of suspension cultures.

2. Rapid freezing method:

This technique is quite simple and involves plunging of the vial containing plant material into liquid nitrogen. During rapid freezing, a decrease in temperature -300° to -1000°C/min occurs. The freezing process is carried out so quickly that small ice crystals are formed within the cells. Further, the growth of intracellular ice crystals is also minimal. Rapid freezing technique is used for the cryopreservation of shoot tips and somatic embryos.

3. Stepwise freezing method:

This is a combination of slow and rapid freezing procedures (with the advantages of both), and is carried out in a stepwise manner. The plant material is first cooled to an intermediate temperature and maintained there for about 30 minutes and then rapidly cooled by plunging it into liquid nitrogen. Stepwise freezing method has been successfully used for cryopreservation of suspension cultures, shoot apices and buds.

4. Dry freezing method:

Some workers have reported that the non-germinated dry seeds can survive freezing at very low temperature in contrast to water-imbibing seeds which are susceptible to cryogenic injuries. In a similar fashion, dehydrated cells are found to have a better survival rate after cryopreservation.

Storage:

Maintenance of the frozen cultures at the specific temperature is as important as freezing. In general, the frozen cells/tissues are kept for storage at temperatures in the range of -70 to -

196°C. However, with temperatures above -130°C, ice crystal growth may occur inside the cells which reduces viability of cells. Storage is ideally done in liquid nitrogen refrigerator — at 1 50°C in the vapour phase, or at -196°C in the liquid phase.

The ultimate objective of storage is to stop all the cellular metabolic activities and maintain their viability. For long term storage, temperature at -196°C in liquid nitrogen is ideal. A regular and constant supply of liquid nitrogen to the liquid nitrogen refrigerator is essential. It is necessary to check the viability of the germplasm periodically in some samples. Proper documentation of the germplasm storage has to be done.

The documented information must be comprehensive with the following particulars:

i. Taxonomic classification of the material
ii. History of culture
iii. Morphogenic potential
iv. Genetic manipulations done
v. Somaclonal variations
vi. Culture medium
vii. Growth kinetics

Thawing:

Thawing is usually carried out by plunging the frozen samples in ampoules into a warm water (temperature 37-45°C) bath with vigorous swirling. By this approach, rapid thawing (at the rate of 500- 750°C min^{-1}) occurs, and this protects the cells from the damaging effects ice crystal formation.

As the thawing occurs (ice completely melts) the ampoules are quickly transferred to a water bath at temperature 20-25°C. This transfer is necessary since the cells get damaged if left for long in warm (37-45°C) water bath. For the cryopreserved material (cells/tissues) where the water content has been reduced to an optimal level before freezing, the process of thowing becomes less critical.

Re-culture:

In general, thawed germplasm is washed several times to remove cryoprotectants. This material is then re-cultured in a fresh medium following standard procedures. Some workers prefer to directly culture the thawed material without washing. This is because certain vital substances, released from the cells during freezing, are believed to promote in vitro cultures.

Measurement of survival/viability:

The viability/survival of the frozen cells can be measured at any stage of cryopreservation or after thawing or re-culture.

The techniques employed to determine viability of cryopreserved cells are the same as used for cell cultures .Staining techniques using triphenyl tetrazolium chloride (TTC), Evan's blue and fluorescein diacetate (FDA) are commonly used.

The best indicator to measure the viability of cryopreserved cells is their entry into cell division and regrowth in culture. This can be evaluated by the following expression.

$$\frac{No.of\ cells/organs\ growing}{No.of\ cells/organs\ thawed} \times 100$$

Plant regeneration:

The ultimate purpose of cryopreservation of germplasm is to regenerate the desired plant. For appropriate plant growth and regeneration, the cryopreserved cells/tissues have to be carefully nursed, and grown. Addition of certain growth promoting substances, besides maintenance of appropriate environmental conditions is often necessary for successful plant regeneration.

Applications of Germplasm Storage:

The germplasm storage has become a boon to plant breeders and biotechnologists.

Some of the applications are briefly described:

1. Maintenance of stock cultures: Plant materials (cell/tissue cultures) of several species can be cryopreserved and maintained for several years, and used as and when needed. This is in contrast to an in vitro cell line maintenance which has to be sub-cultured and transferred periodically to extend viability. Thus, germplasm storage is an ideal method to avoid sub-culturing, and maintain cells/ tissues in a viable state for many years.
2. Cryopreservation is an ideal method for long term conservation of cell cultures which produce secondary metabolites (e.g. medicines).
3. Disease (pathogen)-free plant materials can be frozen, and propagated whenever required.
4. Recalcitrant seeds can be maintained for long.
5. Conservation of somaclonal and gametoclonal variations in cultures.
6. Plant materials from endangered species can be conserved.
7. Conservation of pollen for enhancing longevity.
8. Rare germplasms developed through somatic hybridization and other genetic manipulations can be stored.
9. Cryopreservation is a good method for the selection of cold resistant mutant cell lines which could develop into frost resistant plants.
10. Establishment of germplasm banks for exchange of information at the international level.

Limitations of Germplasm Storage:

The major limitations of germplasm storage are the expensive equipment and the trained personnel. It may, however, be possible in the near future to develop low cost technology for cryopreservation of plant materials.

Commercialization of Plant tissue culture technology-

During the past 20 years Biotech was developed into an independent and maturing industry. There are about 5000 biotech companies around the world with market capitalization of about US$ 200 billion with an annual sales of US 50 $ billion. The companies involved in micro propagation are SPIC, TATA, AVT, Jain drips has earned a lot of money from this. AVT started in 1987 for commercial production of large scale of tissue culture plants and that is the first company followed by Indo American during 1988.

According to the Bioteh Consortium India limited, at the end of 1996 about 76 commercial micro propagation units have registered for the production with the Government of India of which 30 are functional within the country. Totally 40 million plants annually are produced against the installed capacity of 110 million plants.

National facility for Virus diagnosis and Quality Control of Tissue culture raised plants has been opened with main centre at IARI, New Delhi and five satellite centres at National Chemical Lab, Pune, Institute of Himalayan Bioresources Technology, Palampur, IIHR, Bangalore and SPIC, Chennai. The Maharashtra state is advancing in cultivating TC plants.

Many crop technologies have been standardized remains unexplored in Tissue culture. If tissue culture plants are released to meet the demands, the production level improves along with improvement in quality. Many promising varieties have been identified in banana, sugarcane, cardomum, papaya, vanila, gerbera, chrysathemum, orchids etc which are in heavy demand in commercial market can be micropropgated and was multiplied.

The scope of few crops which can be commercially explored by tissue culture are discussed.

Cardamom

India is the land of spices wherein the queen of spices the cardamom are cultivated in an area of 72,400 to 1,02, 400 ha. Cardamom is cultivated for its essential oils and oleoresin apart from it being a spice crop. Tamilnadu and Kerala are the main states that cultivate cardamom. In Tamilnadu, it is cultivated in areas like Kodaikanal hills, cumbum foothills and bodi etc. The

dreaded diseases like katte, kokkekandu, necrosis, soft rot, clump rot and pests like thrips, white flies, root grubs, shoot borers etc cause severe yield loss.

The estimated Global consumption is 15000- 24000 tonnes of which majority comes from Gautemela. Cardamom has a tremendous export potential to countries like Saudi which are the largest consumer in the world followed by India. Out of 15, 250 tonnes produced nearly 7000 tonnes were exported while the rest is used for domestic purposes. Hence this has a potential for both export and domestic uses.

Commonly cultivated varieties include PV 1, CCS 1, ICRI 1, ICRI 2, ICRI 3, Mudigere 1, Mudigere 2 and PV 2 (bold seeded). The varieties like Njallam Green Gold which revolutionized the production and. Sources of resistance is reported to diseases like kokke kandu in the clone 893, for katte disease in NKE 3, NKE 73, for Rhizome rot in RR1 and for drought in P3, P6. All these varieties can be propagated by tissue culture methods. Successful micropropogation is achieved by culturing immature inflorescence as explants. Both large and small cardamom can be propagated by tissue culture methods. The productivity in India is only 250 kg /ha while in country s like Guatemala it is around 200 kg/ha. This may happen because of the diseases and pests that are mentioned. The yield level also increased upto 63. 5 %, which is higher than the cultivation of conventionally propagated plantlets. The tissue culture plants produced in Cardamum will be disease free and the yield level will also be increased.

Vanila

Vanila is cultivated in temperate orchards of Tamilnadu and Kerala. Due to its high rate in the marke tand increased demands to meet food and confectionary industries, the area under Vanila increased tremendously. But diseases like Fusarium rot affected the Vanila plantation drastically. In Kerala, various land races of Vanila are vanished due to the infestation of diseases like wilt and rot. It has been proved that they can be cultivated extensively in the shade of coconut plantations along Pollachi and Palani foothill and even in plains under polyhouses with perfect care. Shoot tips and meristem tips serve as a good explant for propagation. Vanila can be

multiplied, hardened and distributed for commercial plantations based on demand by tissue culture technology, which are free from wilt and other diseases.

Cutflowers

Certain type of flowers is grown as cutflowers because of their special features, particularly long stem or stalk. For example, rose, carnation, gerbera, gladiolus, Chrysanthemum, tuberose, anthurium, etc. There is also varietial preference for them according to the choice of consumers. The cutflowers apart for its domestic use it is also a good foreign exchanger.

In modern "Hi-tech" method the cutflowers are grown in polyhouses/greenhouses requiring high capital investment. But the quality of flowers produced is superior, because inside climate or micro-climate such as temperature, humidity, light, ventilation etc is controlled. Even water application is also controlled. Even water application is also controlled. Therefore, the quality of flowers is better. They are uniform in size, colour, freshness etc. Moreover flowers can be produced throughout the year to meet the market demand-domestic as well as foreign. Since flowers are of better quality, they fetch higher prices

Final consumer use of cutflowers is different from other flowers. Their use is of more sophisticated nature in educated and well to do segment of consumers. Cutflowers are mainly used for preparing bouquets, which are used in functions and ceremonies to welcome guests, VIPs and to felicitate great utility and hence fetch high prices.

One of the study stated that the cutflowers could be produced with a net profit of Rs. 3.59 as a net profit. When gerberas are cultivated the cost of cultivation per flower will approximately Rs.1.04 and the flower can be sold at the price of Rs.2.50 while the net profit will be Rs. 1.46.

Sugarcane

According to the market sources higher cane prices, payment by mills and favorable planting conditions due to late season rains in 2005, it was expected to have an increased area in sugarcane. Industry sources expected 4.4 million hectares under sugarcane production, which is higher than the previous year. Trade sources expected that during 2006-07 sugarcane production might reach at 288 million tons, an increase of 8 per cent over previous year. It also reported that India exported about 1,48,000 tons of sugar during February 2006. Most of these exports were to markets like Pakistan, Bangladesh, and Sri Lanka at cost and freight prices ranging from $440 to $480 per ton. Further the petroleum ministry announced plans to supply ethanol-

blended gasoline across the country this year. For five per cent blending, this would require about 500, 00 kilolitres of ethanol. Most mills prefer to supply molasses directly to the alcohol industry due to better returns. This shows the huge demand of sugarcane and the increase in productivity is essential for the present time. The production and productivity improves by decreasing the incidence of diseases like red rot or any other pests and diseases.

In Tamilnadu an approximately 4.4 million hectares are cultivated by sugarcane and 48 mills (both private and others) are at present and each mill requires 2500 t / day for its crushing with a full capacity. The production decline after 2001-02 and now it is around 234.5 m tonnes. Though India is second in sugarcane area and production, it is 34th in productivity. Red rot is a menace in general and this makes a yield reduction to a maximum.

Tissue culture plants are of tremendous use in vegetatively propagated crops like sugarcane that will improve the yield levels by reducing the incidence of this disease. The sugar mills require 75 tonnes per mill @15 plantlets (@ 15000 plantlets per ha) in the form of breeder seed (setts). These breeder setts will be raised in primary nursery by the mill and will be multiplied in the farmer's field with seven-fold increase each time in the secondary nursery followed by tertiary nursery. These breeder seeds can be supplied to the required mills as tissue culture plantlets.

Successful shoot formation is observed after 8 days of inoculation using Thiodiazuron (TDZ) for micropropagation. There was also an increase of 10- 15 % increase in tissue culture plants. The varieties namely Co 86032, Co 86027, Co 8021, Co 8011, Co, Co 8014, Co 91010, Co 6304, Coc 671, Co v 92102, Co c 92061, Co, Co,

Bamboo

Now a days Bamboos uses in present day life becomes increasing. Besides using it as a building material it is used for paper making, making dress materials like shirts, socks, sarees etc and as edible food. In India, This is cultivated in an area of 9 .57 ha and this covers 12.85 of our forest area. Bamboo grows faster than any other forest trees. Reports are there that the species *Dendrocalamus giganticus* can grow even upto a height of 3 feet per day. Totally 200 plants are needed to plant one hectare. Auxiallary buds can be used as successful explants. This is highly useful in afforestation programme. Introduction and cultivation of thornless bamboo

will be more effective to the end users and propogating them by Tissue culture would also help affoerstation tropical areas where bamboos are not cultivated.

Stevia

The stevioside is a good alternate for sugar and can be used in the place of sugar but without lesser calories than the cane sugar. This stevioside is 20 to 30 times sweeter than cane sugar. This is caloree free sweetener of high quality. 50 grams of stevia leaf replaces 1000 grams of cane sugar. One kg of dried leaves of stevia replaces 20 kg of cane sugar. In India it is estimated that 30 million people are diabetic and for them such sweeteners are the only alternative. The soft drink companies depend on this product. Besides this has antifungal and anti bacterial properties and maintains BP and weight in human.

Banana

Lot of Banana varieties are cultivated Tissue culture banana are marketed by various companies like SPIC, Jain drips etc. The variety Grand nine is most popularly grown in south India. These plants are preferred due to its uniform maturity, improved yield, quality fruits, uniform sized fruits etc. The hill banana is revamped by the tissue culture techniques, Production of virus free stock was made possible in this variety and was reintroduced in their native hilly regions.

So there is plenty of opportunities for cultivation with tissue culture plants

Cost effective techniques in plant tissue culture

The tissue culture technique is not so rigid. The constituents may be altered with the available nutrients, hormones or chemicals. It is essential to standardize according to the needs and targets. However low cost techniques or cost effective techniques will be helpful.

Some of the cost effective techniques are

1. Efforts may be taken to produce more number of plants per unit area
2. The media may be prepared with more care in sterile conditions in order to avoid the wastage of plant materials and media.
3. Always use brands of quality chemicals so as to avoid repletion during preparation of media
4. Low cost hardening materials like compost may be used instead of high cost artificial media
5. Drip fertigation may be practiced inside the green house during hardening
6. Disease free stocks may be used fro effective production of plants
7. Planning may be done to achieve targeted quantities of Tissue culture plants

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