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Plant Tissue Culture- A New Tool for Vegetable Improvement (Indian scenario): A Review

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ABSTRACT

In vitro culture of plant cells/tissues is now routine using a range of explant types from many of the important vegetable and fruit crops. Successful technologies include isolation, culture of tissues, cells, protoplasts, organs, embryos, ovules, anthers and microspores and regeneration from them of complete plantlets. The development of plant tissue culture technology represents one of the most exciting advances in plant sciences. For example, the prospect of being able to introduce, develop, produce, transfer and conserve the existing gene pool of plant sciences by using tissue culture methods opens up new opportunities for researches and entrepreneurs. The term plant tissue culture should denote *in vitro* cultivation of plant cells or tissues in an unorganized mass, *i.e.*, callus culture. Plant tissue culture techniques, in combination with recombinant DNA technology, are the essential requirements for the development of transgenic plants. However, culture techniques like anther/pollen/ovule culture, meristem culture can themselves be utilized for crop improvement or may serve as an aid to conventional breeding. In recent, isolated microspore culture has developed as a breeding tool and an experimental system for various genetic manipulations. The inherent potentiality of a plant cell to give rise to a whole plant, a capacity which is often retained even after a cell has undergone final differentiation in the plant body, is described as 'cellular totipotency'. On the other hand, production of virus-free plants *via* meristem culture can reduce losses caused by phyto-pathogens. Embryo culture has many potential uses ranging from overcoming seed dormancy to facilitation of inter-specific hybridization. Protoplast fusion technique can be used for the transfer of cytoplasmic male sterility from one species to another in a short period of time. In cabbage, male sterile cybrids are being utilized by seed companies to produce hybrid seeds on commercial scale and at competitive rates. Plant tissue culture and cell culture are providing useful methods for germplasm storage either by low temperature storage of organized tissue, or cryopreservation of cell or embryo culture.

Key words: Cell culture, *In vitro*, Microspore culture, Totipotency.

Plant tissue culture can be defined as the culture of all types of plant cells, tissues and organs under aseptic conditions. Plant tissue culture has an important role to play in the manipulation of plants for improved agronomic performance. Plant tissue culture is an integral part of molecular approaches to plant improvement and acts as an intermediary whereby advances made by the molecular biologists in gene isolation and modification are transferred to plant cells. The basic principle of plant tissue culture is totipotency *i.e.*, the capacity of a plant cell to regenerate into a whole plant (Bhojwani and Razdan, 1996.). Some of the simpler techniques that are more approachable and have been found to be applied directly in plant propagation and genetic improvement of plants are (a) micro-propagation, (b) meristem culture, (c) somatic embryogenesis, (d) somaclonal variation, (e) embryo culture, (f) *In vitro* selection, (g) anther culture and (h) protoplast culture (i) cryo-preservation.

History of tissue culture

The history of plant tissue culture dates back at least to 1902, when Gottlieb Haberlandt (Haberlandt, 1902), a German botanist, proposed that single plant cells could be cultured *in vitro*. In 1962, Toshio Murashige and Skoog (Murashige and Skoog, 1962) published the composition a plant tissue culture medium known as *MS* (named for the first letters of their last names) *medium*, which now is the most widely used medium for tissue culture. Commercial tissue culture was born in India in 1987 when A.V. Thomas and Company Kerala (AVT) established their first production unit in Cochin for clonal propagation of superior genotypes of selected cardamom plants. In 1988, a second company Indo-American Hybrid Seeds at Bangalore, Karnataka, who were in the nursery business in hybrid flowers and vegetables, imported a tissue culture laboratory and green houses with a capacity of 10 million plants/ annum.

Media and culture conditions

In addition to MS, there are a plethora of media formulations

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that are available for plant tissue culture. The choice of medium is typically determined empirically for optimal response of the plant species and the research goal. Most media are adjusted to a pH of 5.2-5.8. Successful tissue culture requires the maintenance of a sterile environment. All tissue culture work is done in a laminar flow hood. It is imperative to maintain axenic conditions throughout the life of cultures. For sterilization of small quantities of medium a pressure cooker, which works on the same principle as an autoclave, may be used.

Production of pathogen free plants (meristem culture)

Pests and pathogens cause considerable damage and economic losses to agricultural and horticultural crops. A good amount of this damage is caused by virus infections. The crop plants which are generally propagated by vegetative means, are particularly prone to losses caused by viruses that are transmitted from generation to generation. A rough estimate of annual global losses of agricultural produce by virus infection is to the tune of US$ 6 9 1010 billion (http://www.microbiologybytes.com/virology). Virus infection is also known to reduce the rate of clonal propagation. Control of viral diseases is a serious problem as commercial chemical control methods are either not available or are not economical.

Traditionally, thermotherapy of the infected plants has been used to obtain virus-free plants. However, it is not only a cumbersome process but also not all viruses are eliminated by this method and many host plants are thermo-sensitive. Morel and Martin (1952) further refined the technique and developed the meristem-tip culture technique to eliminate viruses. This *in vitro* technique soon became the most popular technique to eradicate virus from infected plants and is being widely used for the purpose. The distribution of viruses in plants is uneven. The apical meristems of infected plants are either free or carry a very low concentration of viruses (Quak, 1977; Wang and Hu, 1980).

Incorporation of hi-tech seed production system coupled with advance virus detection techniques is the only way out in fulfilling the huge demand of quality seed potatoes in the country. Keeping that in view, ICAR-CPRI, Shimla has standardized a number of high-tech seed production systems based on tissue culture and micro-propagation technologies. The latest hi-tech seed production system standardized by the institute is based on the concept of soil-less, aeroponic technology. ICAR-CPRI produces ~ 3,187 metric tonnes of nucleus and breeder seed of 25 popular potato varieties; out of which 70% is through conventional system whereas, 30% through high-tech systems (Source: ICAR-Central Potato Research Institute, Shimla, 2018).

Production of haploids and double haploids

The technique was discovered by Guha and Maheshwari (1964) from anthers of *Datura* cultured *in vitro*. The technique of haploid production through anther culture has been extended successfully to numerous plant species, including many economically important plants, such as vegetables. During the last decade considerable success has been achieved with the induction of androgenesis in isolated pollen culture and androgenic haploids have been used to breed new cultivars of crop plants.

An isolated microspore culture provides an excellent system for the study of microspore induction and embryogenesis, provides a platform for an ever-increasing array of molecular studies and can produce doubled haploid (DH) plants, which are used to accelerate plant-breeding programs. Moreover, isolated microspore cultures have several advantages over anther culture, wherein presence of the anther walls can lead to the development of diploid, somatic calli and plants. For some of these species, isolated microspore culture protocols are well established and routinely used in laboratories around the world for developing new varieties, as well as for basic research in areas such as genomics, gene expression and genetic mapping. Over the past few years, a large proportion of the research reports on isolated microspore culture have focused on cereal and *Brassica* species (Ferrie and Caswell, 2011).

Embryo rescue

Several physical and chemical factors regulate the growth and development of the embryo. The surrounding tissues, especially the endosperm, also control the predetermined pattern of embryo development. Any disturbance in these factors causes abnormalities and, in extreme cases, abortion of the embryo. *In vitro* culture of excised zygotic embryos at different stages of development has provided useful information on the developmental and physiological aspects of embryogenesis. This technique of hybrid embryo culture has since been widely used to raise numerous such hybrids which normally fail due to post-zygotic sexual incompatibility (Bhojwani and Dantu, 2012). The first embryo culture was done by Hanning (1904) from two crucifers *Cochleria* and *Raphanus*.

Somatic hybridization

Protoplasts are single cells that have been stripped of their

Current Role of Tissue Culture in Crop Improvement.

Technology	Application
Meristem and bud culture :	Micropropagation for commercial purposes, genetic conservation genetic transformation and exchange of material
Anther and microspore culture:	Haploid and double haploid production
Zygotic embryo culture:	Interspecific crosses
Cell and tissue culture:	*In vitro* selection, somaclonal variation, somatic embryogenesis, artificial seeds
Protoplast culture:	Fusion for somatic hybridization

wall exposing the plasmalemma. One gram of leaf may yield about a million protoplasts. A special property of the protoplasts is that when brought into close contact they tend to fuse with each other irrespective of the sources of the protoplasts. The technique of fusion of isolated protoplasts from somatic cells and regeneration of hybrid plants from the fusion products, called somatic hybridization, completely bypasses the sex and thus allows combining genomes of two desirable parents, irrespective of their taxonomic relationship (Bhojwani and Dantu, 2013). Somatic hybridization was first reported by Carlson *et al*. (1972). Tissue culture-induced somaclonal variation is akin to variations induced with chemical and physical mutagens (Jain, 2001) and offers an opportunity to uncover natural variability for their potential exploitation in crop improvement.

Somaclonal variation

The terminally differentiated cells in plants, such as pith cells and cortical cells, often exhibit cytological and genetic changes that go unnoticed and do not cause abnormalities in the plant because they do not form part of the germ line cells. However, in cultures these cells may be induced to undergo redifferentiation and express the inherent variability at the whole plant level. Additional variations may be induced by the culture conditions. While some of these variations aretransient physiological and developmental changes (*e.g.*, rejuvenation), others are a result of epigenetic changes which can be relatively stable but are not transmitted to the seed progeny. Some other *in vitro* induced variations are caused by specific genetic changes or mutations and are transmitted to the progeny. The genetic and epigenetic changes observed in plants regenerated from cultured somatic cells are referred to as somaclonal variation (soma = vegetative, clone = identical copy), a term coined by Larkin and Scowcroft (1981).

Somatic embryogenesis

Somatic embryogenesis a nonsexual developmental process that produces a bipolar embryo with a closed vascular system from somatic tissues of a plant. Somatic embryogenesis has become one of the most powerful techniques in plant tissue culture for mass clonal propagation. Somatic embryogenesis may occur directly or via a callus phase. Direct somatic embryogenesis is preferred for clonal propagation as there is less chance of introducing variation *via* somaclonal mutation. Indirect somatic embryogenesis is sometimes used in the selection of desired somaclonal variants and for the production of transgenic plants.

Large scale production of somatic embryos using bioreactors and synthetic seeds from somatic embryos has been successful. Somatic embryos can be cryopreserved as synthetic seeds and germinated whenever necessary. One advantage of somatic embryogenesis is that somatic embryos can be directly germinated into viable plants without organogenesis; thus, it mimics the natural germination process (Cardoza, 2016).

Synthetic seed

Encapsulated somatic embryos are known as *synthetic seeds*. Somatic embryos are typically encapsulated in an alginate matrix, which serves as an artificial seed coat. The encapsulated somatic embryos can be germinated *ex vitro* (“out of glass”) or *in vitro* to form plantlets. Synthetic seeds have multiple advantages-they are easy to handle, they can potentially be stored for a long time and there is potential for scale up and low cost of production. Another advantage is the prospects for automation of the whole production process because the commercial application of somatic embryogenesis requires high volume production. Synthetic seeds can be stored at 4°C for shorter periods or cryopreserved in liquid nitrogen for long term storage (Fang *et al*., 2004).

Organogenesis

Organogenesis is the formation of organs: either shoot or root. Organogenesis *in vitro* depends on the balance of auxin and cytokinin and the ability of tissue to respond to phytohormones during culture. Organogenesis takes place in three phases. In the first phase, the cells become competent; next, they dedifferentiate. In the third phase, the tissues redifferentiate and morphogenesis proceeds according to the developmental program, which ultimately produce whole intact plants (Sugiyama, 1999). *In vitro* organogenesis can be of two types: direct and indirect.

Indirect Organogenesis

The formation of organs indirectly via a callus phase is termed *indirect organogenesis*. Induction of plants using this technique does not ensure clonal fidelity, but it could be an ideal system for selecting somaclonal variants of desired characters and also for mass multiplication. Induction of plants *via* a callus phase has been used for the production of transgenic plants in which (a) the callus is transformed and plants regenerated or (b) the initial explant is transformed and callus and then shoots are developed from the explant.

Direct organogenesis

The production of direct buds or shoots from a tissue with no intervening callus stage is termed *direct organogenesis*. Plants have been propagated by direct organogenesis for improved multiplication rates, production of transgenic plants and-most importantly-for clonal propagation. Typically, indirect organogenesis is more important for transgenic plant production. Axillary bud induction/multiple bud initiation is a set of techniques that represent the most common means of micro-propagation since it ensures the production of uniform planting material without genetic variation. Axillary shoots are formed directly from preformed meristems at nodes and the chance of the organized shoot meristem undergoing mutation is relatively low. This technique is often referred to as multiple bud induction. (Cardoza, 2016).

Long term storage of tissues

Long-term preservation of germplasm is through cryopreser-

-vation, in which the material is frozen at the temperature of liquid nitrogen (-196°C). At this super-low temperature, all metabolic activities of the cells are suspended. Therefore, in theory, the cryopreserved cells can be stored for unlimited period without a subculture. However, adequate care is required to protect the cells from cryogenic injury during freezing, storage and retrieval. During the past three decades, considerable work has been done to develop effective protocols for cryopreservation of plant cells, tissues and organized structures (shoot tips, embryos *etc*.) and the technique is being increasingly used for conservation of germplasm (Ashmore, 1997; Benson, 1999; Engelmann, 2011; Gonzalez Arnao *et al*., 2008). The number of plant species for which cryopreservation protocols have been established is over 200 (Engelmann, 2004). Some of the centers where germplasms have been maintained in liquidnitrogen are: International Potato Centre, Peru, Laboratory of Tropical Crop Improvement, Belgium, National Seed Storage Lab, USA, ORSTOM, France and Tissue Culture Research Inc., Canada (5000 accessions of conifer species). In India, NBPGR (New Delhi) and TBGRI (Thiruvenanthapuram)are working on cryopreservation of germplasm.

The main consideration in cryopreservation is to protect the cells by preventing or minimizingice crystal formation during cooling. This has led to two main approaches to cryopreservation of plant materials: (1) Slow or Step-wise freezing method (also known as the traditional method) and (2) Ultra-rapid freezing method, which is comparatively new and is becoming increasingly popular. In both the methods, cells are sufficiently dehydrated during or before freezing, so that the protoplasm becomes concentrated and ice crystal formation is prevented. A pre-culture step is generally helpful in enhancing the desiccation tolerance of the cells. There are four main steps in cryopreservation of plant materials: (1) Pre-culture, (2) Freezing, (3) Thawing and (4) Re-culture. The choice of the tissue also contributes toward the success of a cryopreservation protocol.

In vitro maintenance of plant organs can enhance programs in plant breeding and germplasm resources. In bulbous plants, such as onion (*Allium cepa* L.) and leek (*Allium ampeloprasum* L.) induction and storage of in vitro bulblets could enable long term maintenance of special genotypes. *In vitro* cultivated seedlings of onion and leek were induced to form bulblets by increasing the source concentration and treatment with ethephon. Bulblets obtained were used for *in vitro* storage under conditions of slow growth (Keller, 1993). The influence of light conditions, sucrose and ethylene on *in vitro* formation and storability of onion (*Allium cepa* L.) bulblets were studied in various accessions by Kastner *et al*. (2001). They opined that storability was primarily enhanced by a high sucrose concentration (100 g/l) in culture medium. Being seed propagated onion is not stored under liquid nitrogen commercially.

Indian scenario vs rest of the world in tissue culture

The Indian scenario of tissue culture industry clearly indicates that it is a flourishing industry with about 125 tissue culture units with a total production capacity of 300 million plants per annum currently. Vegetables like tomato and potato, spices like cardamom, turmeric, ginger, pepper are being produced. In India that about 125 commercial units are functioning, while Indonesia and Japan have 33 units each, Korea has 20 and Thailand has 18 units. Other global countries involved in tissue culture plants production include European countries, USA, Canada, Australia, New Zealand, Israel, Middle East, South and Central America and Africa. All these countries together produced about 900 million plants in the year 2003.

CONCLUSION

Plant tissue culture represents the most promising areas of application at present time and giving an out look into the future. The global biotech business is estimated about 50 billion out of which 10% is the contribution of plant tissue culture products. Advances made in commercialization of plant tissue culture and the acceptance of tissue culture-derived plantlets by commercial nursery traders globally has led to the growth of this industry. In *vitro* screening could help in isolating new and improved cell lines, from which plants with improved traits can be regenerated.

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