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Plant Tissue Culture Technique as a Novel Tool in Plant Breeding: A Review Article

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Abstract: As enabling and emerging technology, plant tissue culture techniques have been developed and used as a novel tool to assist plant breeders in crop improvement perspectives. These novel tools can be used to either increase the speed and/or the efficiency of breeding process in order to improve the accessibility of existing germplasm and to create new genetic variation for crop improvement as well as to be able to achieve the objective which is not possible through conventional breeding methods. These include eradication of pathogens from planting materials, removal of sexual incompatibility by embryo rescue technique, somatic hybridization through protoplast technology, gene transformation in transgenic technology, production of haploids via anther culture and most importantly the induction of new genetic variability through somaclonal variation and selection of desirable agronomic traits. Thus, plant tissue culture technology has a vast potential to produce plants of superior quality and selection of useful variants in well adapted high yielding genotypes with better disease resistance and stress tolerance capacities.

Key words: Embryo rescue · Germplasm conservation · Haploids · Pathogen eradication · Protoplast fusion · Somaclonal variation · Synthetic seed

INTRODUCTION

Conventional plant breeding can no longer sustain the global demand with their increasing population, decline in agricultural resources such as land and water and the apparent plateauingof the yield curve of the staple crops. Thus, new approach for crop improvement technologies has to be developed and utilized for plant breeders to overcome these problems. Extensive research has resulted in new areas of plant breeding, namely "plant biotechnology" and "genetic engineering". They are based on cellular totipotency or the ability to regenerate the whole flowering plants [1, 2, 3].

Some plants and trees need several years before they flower and set seeds, making plant improvement difficult. Some crops including banana, apple, cassava and sugarcane reproduce vegetatively, especially those that are fully sterile without seeds. For this important group, alternative approaches had to be developed, namely techniques of manipulation with somatic tissue: mutation breeding and biotechnology. Thus, scientists have developed the science and art of plant tissue culture to assist breeders in this regard. It is well known that the process of developing new crop varieties through conventional plant breeding requires many steps and can take almost 7-10 years [2]. Now, however, applications of plant tissue culture technology have considerably shortened the time it takes to bring them to market. Under the right conditions, an entire plant, large amounts of new cells or tissue can be regenerated from a single cell. Moreover, in certain situations, the conventional methods have to be supplemented with plant tissue culture techniques either to increase their efficiency or to be able to achieve the objective which is not possible through the conventional breeding methods [2]. Thus, application of plant tissue culture represents the most promising area at present time and giving an outlook and more emphasis in the future science-based agricultural research. Since the application areas ranges from micropropagation of ornamental and forest trees, production of pharmaceutically valuable compounds and crop breeding for improving nutritional value of staple crop plants, including cryopreservation of valuable germplasms [3]. Thus, it is now the most an efficient technology for crop improvement program through generating new genetic variants which is a prerequisite for

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any crop improvement work. This technology has various potential applications interms of producing plants with superior quality and identification of useful variants in well adapted high yielding genotypes with better biotic and abiotic stress tolerant capacities [4]. These somaclonal variants have been developed in tissue culture processes and give rise to clones that have inheritable characteristics different from those of parent plants due to the possibility of somaclonal variability [5], which leads to the development of commercially important improved varieties.Further, tissue culture process has also been produced different somaclonal and gametoclonal variants with crop improvement potential since production of mutant lines is highly desirable for plant breeding work.

As reported by Brown and Thorpe new genetic variability has been introduced through tissue culture process from which crop plants can be improved and/or to improve the health of the planting material as well as increasing the number of desirable germplasms available to national breeding programs [4]. Various tissue culture techniques such as culture of protoplasts, anthers, microspores, ovules and embryos have been used to introduce new genetic variation in breeding lines, often via haploid production. The technique is also useful for rescuing embryos from incompatible crosses, shortening the breeding cycle and overcoming seed dormancy. Moreover, the culture of single cells and meristems can be effectively used to eradicate pathogens from planting material and thereby dramatically improve the yield of established cultivars. More recently, in the combination of tissue culture with molecular techniques have been now successfully used to incorporate specific traits through gene transfer [4].

Therefore, the aim of this paper is to present clear novel applications of plant tissue culture technique in plant breeding; through embryo rescue, protoplast fusion, haploid production, generation of pathogen free plant material, synthetic seed production, somaclonal variation and germplasm conservation.

Historical Perspective of Plant Tissue Culture: Plant tissue culture is the science of growing plant cells, tissues, organs, or any plant parts isolated from the mother plant, on artificial media under aseptic conditions (*in vitro*). The science of plant tissue culture takes its roots from path breaking research in biology like discovery of cell followed by propounding of cell theory. It was Gottlieb Haberlandt [1] who in the first decade of this century pioneered the field of plant tissue culture. His idea was to achieve continued cell division in explanted tissue grown on nutrient medium. Though he was laid down the foundation of tissue culture technology for which he is regarded as the father of plant tissue culture [3]. Following the discovery and use of auxins, the work of Gautherel, Nobecourt and White ushered in the second phase of plant tissue culture over 30 years ago. These and other scientists determined the nutritional and hormonal requirements of the cultured plant tissues. Rapid advances in diverse aspect of plant tissue culture have been made during the last few years and plant tissue culture techniques have been extensively applied to agriculture and industry.

Principles of Plant Tissue Culture: As a principle, plant cells, tissues, organs and any other parts of the plant are cultured *in vitro* on artificial nutrient media, under aseptic and controlled environment. It is well known that starting plant materials from the field are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms, thus surface sterilization of starting materials (explants) in chemical solutions (usually alcohol or bleach) is very important. After that the explants are usually placed on the surface of a solid culture medium, but are sometimes placed directly into a liquid medium, particularly when cell suspension cultures are desired.

Plant tissue culture depends mainly on the concept of totipotentiality of plant cells [1] which refers to the ability of a single plant cell to express the full genome by cell division and/or to grow and develop into a fully differentiated plant. In addition to the totipotent potential of plant cell, the capacity of cells to alter their metabolism, growth and development is also equally important to regenerate the entire plant [6]. The culture medium contains all the necessary nutrients required for the normal growth and development of plants. It is mainly composed of macro and micro nutrients, vitamins, amino acids, plant growth regulators, carbon source and some gelling agents in the case of solid culture medium [1]. The pH of the media is also very crucial since it affects both the growth of plants and activity of plant growth regulators [3], thus it has to be adjusted at the critical value. The culture medium composition, specially the plant growth regulators and the nitrogen source has profound effects on the response of the initial explant.

In general, there are a number of tissue culture techniques and they can be employed for different purposes. More recently, plant tissue culture has been given the highest priority in plant breeding work since it provides immense potential for crop improvement programs such as selecting disease/insect, or stress

resistant plants, regeneration of the novel hybrid via protoplast technology, rescue the embryos from wide crosses through embryo culture, haploid and dihaploid production within short time frameetc. [7]. The techniques include; (i) callus cultures, (ii) cell suspension cultures, (iii) protoplast cultures, (iv) meristem cultures, (v) root cultures, (vi) endosperm cultures, (vii) ovule cultures, (viii) anther/pollen cultures, (ix) embryo cultures, (x) seed cultures etc.

Novel Applications of Plant Tissue Culture in Plant Breeding: Recently, plant tissue culture has an indispensable application on both agriculture and industry, through providing plants needed to meet the ever increasing world demand [3]. It has also made significant contributions to the advancement of agriculture in recent times and today they constitute novel tools to assist breeders in modern plant breeding work [8], through production and propagation of genetically homogeneous, disease-free plant material [9] and the induction of somaclonal variation [10]. Since it is now possible to induce new genetic variability through tissue culture and could be used as a source of variability to obtain new stable genotypes [3]. Therefore, the novel applications of plant tissue culture particularly in the area of plant breeding for the sake of crop improvement are listed below:

- Embryo rescue
- Protoplast fusion/somatic hybridization
- Haploid production
- Generation of pathogen-free plant material
- Synthetic seed production
- Somaclonal variation
- Germplasm conservation

Embryo Rescue: Wide hybridization crosses can result in small shrunken seeds which indicate that fertilization has occurred, however the seed fails to develop further. In most instance, wide hybridizations will fail to undergo normal sexual reproduction, thus embryo rescue can assist in circumventing this problem. Embryo rescue is one of the earliest and successful forms of tissue culture techniques that are used to assist in the development of plant embryos that might not survive to become viable plants and mostly used to develop interspecific and intergeneric crosses that would normally produce seeds which are aborted. Thus, embryo rescue plays a vital role in modern plant breeding through the development of many interspecific and intergeneric crop hybrids. Among other reasons, embryo abortion is considered to be the major reason for interspecific incompatibility in plants. Therefore, embryo culture technique has been applied successfully in overcoming this major problem as well as solving the problems of low seed set, seed dormancy and slow seed germination [4]. Currently, a number of interspecific and intergeneric hybrids of agriculturally important crops have been successfully developed through embryo culture technique, including cotton, barley, tomato, rice, jute, *Hordeum*x *Secale, Triticum*x *Secale, Tripsacumx Zea* and some *Brassica* species [11, 12]. Therefore, in general embryo culture technique enables the breeder to successfully make wide crosses with a greater number of related species of wild plants and have access to a much wider range of genes that can be used for genetic improvement of crop plants.

Major Applications of Embryo Culture Techniques

Prevention of Embryo Abortion in a Wide Cross: In interspecific and intergeneric hybridization programs, incompatibility barriers often prevent normal seed development and production of hybrids. Although there may be normal fertilization in some incompatible crosses, embryo abortion results in the formation of shriveled seeds. Poor and abnormal development of the endosperm caused embryo starvation and eventual abortion. Thus, isolation of hybrid immature embryos before abortion and culture *in vitro* system may prevent these strong post-zygotic barriers [13]. The most useful and popular application of embryo cultures is to raise rare hybrids by rescuing embryos of incompatible crosses.

Overcoming Seed Dormancy and Shortening Breeding Cycle: Long periods of dormancy in seeds delay breeding works, especially in horticultural and crop plants. Using embryo culture techniques the breeding cycle can be shortened in these plants [13]. For instance, the life cycle of *Iris* was reduced from 2-3 years to less one year.Similarly, it was possible to obtain two generations of flowering against one in *Rosa* specie. Germination of excised embryo is regarded as a more reliable test for rapid testing of viability in seeds, especially during dormancy period.

Overcoming Seed Sterility: In early ripening fruit cultivars, the seed do not germinate because their embryos are still immature. Using the embryo culture method it is possible to raise seedling from sterile seeds of early ripening of stone fruits, peach, apricot and plum. For instance, ‘Makapuno’ coconunts are very expensive

and most relished for their characteristics of soft fatty endosperms in place of liquid endosperm [13]. Under normal conditions the coconut seeds fail to germinate. However, it is possible to obtain successful germination in raising field grown makapuno trees with the aid of embryo culture technique.

Production of Monoploid/Haploid: An embryo culture has been used in production of monoploids of barley. With the cross *Hordeum vulgare*, fertilization occurs normally but thereafter chromosomes of *H.bulbosum* are eliminated, resulting in formation of monoploid *H. vulgare* embryo which canbe rescued by embryo cultures [4].

Protoplast Fusion/Somatic Hybridization: The incompatibility barriers in sexual recombination at interspecific or intergeneric levels are also overcome by using protoplast fusion technique. Because (i) many desirable combinations of characters cannot be transmitted through conventional breeding methods of genetic manipulation and (ii) conventional hybridization is also limited to only very closely related species and was total failure for distantly related species as well as in sexually incompatible species. However, by using a protoplast fusion technology, it is now possible to fuse two genetically different species by only protoplast to obtain para sexual hybrid protoplast. Thus, protoplast fusion is a novel tool for plant breeding and crop improvement by developing interspecific and/or intergeneric unique hybrid plants which cannot be produced by conventional sexual hybridization [3, 4]. The technique involves the fusion of protoplasts of two different genomes of genetically unrelated species followed by the selection of desired somatic hybrid cells and regeneration of hybrid plants [14]. Protoplast fusion technology is one of the pioneered technologies that provides an efficient ways of gene transfer with desired trait from one species to another and thus has significant contributions on national crop improvement program [4]. Recently, the protoplast has been isolated from numerous crop species like barley, carrot, cassava, cotton, pea and soybean. However, one of the earliest successes in this technology was the pomato (potato-tomato fusion product). In addition to this, intergeneric protoplast fusion has also been made in carrot x petunia, maize x sorghum and soybean x barley [15]. Similarly, Mostageer and Elshihy [16] reported somatic hybrid cells were produced by fusion of protoplasts from rice and ditch reed using electrofusion treatment for salt tolerance.

More recently, protoplast fusion technology opens means of developing unique hybrid plants by solving the problems of sexual incompatibility [3]. It is highly applicable in horticultural industry in order to develop new hybrids with increased fruit yield and better resistance to biotic and abiotic stress. It has also been reported that successful viable hybrid plants were developed when protoplasts from citrus were fused with other related *citrinae* species [17]. In general, according to Evans and Bravo [14] recommendation, production of novel hybrid plants through protoplast fusion technology should focus on four areas: (i) agriculturally important traits; (ii) achieving combinations that can only be made by protoplast fusion; (iii) somatic hybrids has to be integrated into a conventional breeding program; and (iv) the extension of protoplast regeneration to a wider range of crop species.

However, there are various limitations and considerations in this technology which include (1) intergeneric crosses between widely related plants which are sexually incompatible are not possible, (2) in certain wide crosses, elimination of chromosomes from hybrid cell is another limitation, (3) in protoplast fusion experiments, the percentage of fusion between two different parental protoplast is very low and (4) for hybrid identification, selection and isolation at the culture level, there is no standardized and optimized method which is applicable for all material.

Haploid Production: *In vitro* production of haploids can solve some problems in genetic studies of plants since gene action is readily manifested due to a single allelic gene present in chromosome of entire genome. By doubling their chromosomes number, the plants can be made fertile and resultant plants will be homozygous. Thus, tissue culture techniques enable to produce homozygous plants in relatively short time period through protoplast, anther and microspore cultures instead of conventional breeding methods [18].

Haploids are sterile plants having single set of chromosomes (one-half of the normal number of chromosomes) which are converted into homozygous diploids by spontaneous or induced chromosome doubling [3, 12, 19] since the doubling of chromosomes restores the fertility of plants resulting in production of double haploids with potential to become pure breeding new cultivars [20]. Similarly, Brown and Thorpe [4] pointed out that haploids are of interest to plant breeders; (i) because they allow the expression of simple recessive genetic traits or mutated recessive genes which is very

important for selection of desirable traits like resistance to diseases, insects, antibiotics, salts etc have been isolated from haploids derived from anther culture and (ii) because dihaploids which is derived from haploids, can be used immediately as homozygous breeding lines in breeding program. The efficiency in producing completely homozygous breeding lines via doubled *in vitro* produced haploids represents significant savings interms of both time and cost as compared with other conventional methods [4].

In general, the haploidy technology has now become an important integral part of plant breeding programs by speeding up the production of homozygous/inbred lines [21] and overcoming the constraints of seed dormancy and embryo non-viability [22]. The technique has a remarkable use in genetic transformation by the production of haploid plants with induced resistance to various biotic and abiotic stresses. Moreover, Chauhan and Khurana [23] suggested that introduction of genes with desired trait at haploid state followed by chromosome doubling led to the production of double haploids inbred wheat and drought tolerant plants were attained successfully.

Generation of Pathogen Free Plant Material: Another most interesting application for which plant tissue culture is uniquely suited is in the obtaining, maintaining and mass propagating of specific pathogen-free plants by meristem culture technique.This technique was first developed for virus eradication by Morel and Martin [24] on Dahlia and leads to pathogen-free plants. Recently, meristem culture has been used successfully in the removal of viruses from many plants (potato, sugarcane, strawberry) and is now used routinely for the eradication of many viral diseases from plant materials.

Lizarraga *et al*. [25] stated that plant pathogens, such as nematodes, fungi, bacteria and viruses, can be transmitted from diseased plants to healthy plants. However, not all plant cells become infected; the meristematic tissues are sometimes disease-free. It is possible to recover non-infected plants by*in vitro* meristem culture techniques and to grow them into healthy plants.

The distribution of the different pathogens within a diseased plant also varies greatly. Potato leaf roll virus (*Pseudomonas solanacearum*) and mycoplasma are restricted to the vascular tissue of a plant. *Erwinia carotovora* and potato virus X (PVX), invades both vascular and non-vascular plant tissues. Not all the cells in a diseased plant are infected with pathogens. The meristematic tissues of roots and shoots of an infected plant are sometimes free of viruses. In certain cases, such as potato virus X (PVX) and tobacco rattle virus in potatoes, only the apical dome and the first young primordial leaf are free of viruses. The exact reason for this is not known, however, it is believed that one or all the following factors are responsible [25].

- *High metabolic activity*: Viruses replicate by taking over the host metabolic pathways. Due to the high metabolic activity in these cells viruses are unable to take over control of the host biosynthetic machinery.
- *Lack of vascular system*: Viruses spread rapidly through the vascular system. Phloem restricted viruses (PLRV) cannot invade the meristematic tissues due to the absence of cell differentiation.In this meristematic region, viruses which infect non-vascular tissues spread from cell to cell through the plasmodesmata. This is a slow process which makes it relatively difficult for viruses to completely infect the rapidly dividing cells.
- *High auxin concentration*: Plant meristematic tissues have a higher auxin concentration than tissues from the other plant regions. These auxins have been reported to inhibit the replication of viruses.

Therefore, meristematic tissues are sometimes free of pathogens; it is possible to recover non-infected plants by *in vitro* meristem culture techniques and to grow them into healthy plants. In addition, thermotherapy, chemotherapy and surface sterilization applied to the whole plant as well as explants followed by meristem culture has been successfully used for elimination of many viruses in plants [25].

Synthetic Seed Production: As stated by Ara *et al*. [26], synthetic seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under *in vitro* or *ex vitro* conditions and that retain this potential also after storage.

The synthetic seed technology has been developed to use somatic embryos and/or other micropropagules as seed analogues successfully in the field or greenhouse and their mechanical planting at a commercial level. The technology provides methods for preparation of seed analogues called synthetic seeds or artificial seeds from the micropropagules like somatic embryos, axillary shoot buds, apical shoot tips, embryogenic calli as well as protocorm or protocorm like bodies [26]. For the last fifteen years, intensive research efforts have been made

on synthetic seed production in a number of plant species. Despite these research studies, practical implementation of the technology is yet to be fully realized due to limitations encountered with the production, development, maturation and subsequent conversion of the micropropagules into plantlets under *in vitro* or *ex vitro* conditions [26]. However, production of artificial seeds has unraveled new vistas in plant biotechnology. The synthetic seed technology is designed to combine the advantages of clonal propagation with those of seed propagation and storage.

Somaclonal Variation: In nature, the genetic diversity and variability within a population are generated via recombination events [27]. Factors such as natural selection, mutation, migration and population size influence genetic variability in different ways. However, genetic variation arising from tissue culture of plants has been termed somaclonal variation [28]. Variation has been observed in a wide range of species from plants derived from a variety of explants, using different tissue culture techniques.

Crop improvement is amulti disciplinary activity concerned with the optimization of genetic attributes within the constraints of the environment and of environmental factor within the constraints of the genetic material [29]. Conventional breeding exploits the natural variation existing in plant populations to recover elite crops. However, the available genetic variability in gene pools is one of the limits to crop improvement. Conventional breeding in its rnain fold efforts to produce a plant ldeotype exploits this natural variation existing in the base.

The evolution of the theoretical aspect of *in vitro* culture paved the way for the emergence of its practical applications which reached exploding proportions in the past decade or so. Initially used for clonal propagation of plants, it later pioneered several new possibilities like removal of sexual incompatibility by embryo rescue techniques, somatic hybridization through protoplast technology, transgenic plant through genetic engineering, production of haploids via anther culture and most important in the context of the present issues i.e. the induction of new genetic variability and selection of desirable traits like salt tolerance [30], disease resistance [31] and pest resistance [32]. Thus, *in vitro* culture, no longer sacrosanct, has emerged as biotechnological tool to widen the germplasm base.

In vitro culture is a rich source of genetic variation. The best available germplasm may be subjected to a tissue culture cycle with or without selection pressure and regenerants selected for superiority of one or more traits while retaining all the original characters. Such incremental improvement in desirable traits could therefore lead to the formation of new alleles spontaneously generated *in vitro*. Thus, tissue culture techniques leading to somaclonal variation could be capitalized upon to accelerate progress in conventional breeding since in the plant breeder's perspective however, the bottom line, remains ultimately that the genetic variability recovered from tissue culture regenerated plants should result in a phenotype that is agriculturally useful. It has also been most successful in crops with limited genetic systems and/or narrow genetic bases, where it can provide a rapid source of variability for crop improvement.

Germplasm Conservation: According to Sengar *et al*. [33], plant tissue culture technique offers an alternative source for the conservation of endangered genotypes and/or species. Filho *et al*. [34] reported that germplasm conservation is increasingly becoming an essential activity due to the high rate of disappearance of plant species and the increased need for safeguarding the floristic patrimony of the countries. Tissue culture protocols can be used for preservation of vegetative tissues when the targets for conservation are clones instead of seeds, to keep the genetic background of a crop and to avoid the loss of the conserved patrimony due to natural disasters, whether biotic or abiotic stress [35]. The plant species which do not produce seeds (sterile plants) or which have 'recalcitrant' seeds that cannot be stored for long period of time can successfully be preserved via *in vitro* techniques for the maintenance of gene banks [3].

Cryopreservation technique plays a vital role in the long-term *in vitro* conservation of essential biological material and genetic resources. It involves the storage of *in vitro* cells or tissues in liquid nitrogen that results in cryo-injury on the exposure of tissues to physical and chemical stresses. Successful cryopreservation is often ascertained by cell and tissue survival and the ability to re-grow or regenerate into complete plants or form new colonies [36]. It is desirable to assess the genetic integrity of recovered germplasm to determine whether it is 'true-to-type' following cryopreservation. Harding *et al*. [37] also stated that the fidelity of recovered plants can be assessed at phenotypic, histological, cytological, biochemical and molecular levels, although, there are advantages and limitations of the various approaches used to assess genetic stability.

CONCLUSIONS

Plant tissue culture technology has been widely used for improving the most important agricultural crops as well as endangered native species. Somatic hybridization is another way of enhancing variation in crop species by importing genes or even whole chromosomes from other species that are not closely enough related for normal sexual crossing. Although similar in its aims to conventional hybridization, somatic hybridization involves a more radical technological approach. Haploid plants can be produced using anther culture which involves the *in vitro* culture of immature anthers. As the pollen grains are haploid, the resulting pollen-derived plants are also haploid. Doubled haploid plants were first produced in the 1960s using colchicine treatment. Doubled haploids may also be produced from ovule culture. Doubled haploid plants are the so-called breeder's value because they are 100 percent homozygous and any recessive genes are therefore readily apparent. The time required after a conventional hybridization to select pure lines carrying the required recombination of characters is consequently drastically reduced. The application of this technique to plant breeding is hindered by the investments in facilities and human resources necessary to produce and to test large populations of doubled haploids. Manipulations by plant breeders frequently result in sterile varieties from wide crosses and that cannot readily be propagated. Sometimes this is a useful trait and is deliberately engineered by breeders. Thus, chromosome doubling during embryo culture is one of the most important technologies for the creation of fertile interspecific hybrids. Wide hybrid plants are often sterile so their seeds cannot be propagated. This is due to differences between chromosome sets inherited from genetically divergent parental species, which prevent stable chromosome pairing during meiosis. However, if the chromosome number is artificially doubled, the hybrid may be able to produce functional pollen and eggs and therefore be fertile. Somaclonal changes in cultured plant cells can potentially provide a powerful new tool to generate genetic variation for plant breeders. Somaclonal mutagenesis has been used to manipulate traits such as disease resistance, insect resistance, nutritional value, drought and salt tolerance in crop species.

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