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Full Length Research Paper

Tissue culture as a plant production technique for horticultural crops

Akin-Idowu, P. E.[1], Ibitoye, D. O.[1]* and Ademoyegun, O. T.[2]

[1]Fruits and Biotechnology Programme, National Horticultural Research institute, P.M.B. 5432, Idi-Ishin, Jericho, Ibadan, Nigeria.
[2]Crop Utilization Unit, National Horticultural Research Institute, P.M.B. 5432, Idi-Ishin, Jericho, Ibadan, Nigeria.

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Over 100 years ago, Haberlandt envisioned the concept of plant tissue culture and provided the groundwork for the cultivation of plant cells, tissues and organs in culture. Initially plant tissue cultures arose as a research tool and focused on attempts to culture and study the development of small, isolated cells and segments of plant tissues. At the peak of the plant tissue culture era in the 1980s, in a relatively short time, many commercial laboratories were established around the world to capitalize on the potential of micropropagation for mass production of clonal plants for the horticulture industry. Today plant tissue culture applications encompass much more than clonal propagation. The range of routine technologies has expanded to include somatic embryogenesis, somatic hybridization, virus elimination as well as the application of bioreactors to mass propagation. Perhaps the greatest value of these tissue culture technologies lies not so much in their application to mass clonal propagation but rather in their role underpinning developments and applications in plant improvement, molecular biology and bioprocessing, as well as being a basic research tool. Plant tissue culture technique though an underutilized tool in Nigeria, it can be extensively applied in horticulture to increase crop production. This paper highlights some of the applications of plant tissue culture to horticulture, the achievements and limitations of tissue culture and some insights into current and possible future developments. With rapid population growth, the total acreages of fruits, vegetables and various ornamental plants have not been able to meet the needs of people in the developing countries.

Key words: Bioprocessing, clone, micropropagation, somatic embryogenesis, tissue culture.

INTRODUCTION

Plant tissue culture is now a well-established technology. Like many other technologies, it has gone through different stages of evolution; scientific curiosity, research tool, novel applications and mass exploitation. Initially, plant tissue culture was exploited as a research tool and focused on attempts to culture and study the development of small, isolated segments of plant tissues or isolated cells. Around the mid twentieth century, the notion that plants could be regenerated or multiplied from either callus or organ culture was widely accepted and practical application in the plant propagation industry ensued. The technique was heralded as the universal mass clonal plant propagation system for the future and the term 'micropropagation' was introduced to describe more accurately the processes. Many commercial laboratories were established around the world for mass clonal propagation of horticultural plants. Today plant tissue culture applications encompass much more than clonal propagation and micropropagation. The range of routine technologies has expanded to include somatic embryogenesis, somatic hybridization, virus elimination as well as the application of bioreactors to mass propagation. The list includes:

- Clonal propagation
- Axillary shoot multiplication
- Direct (adventitious) organogenesis
- Callus to organogenesis
- Somatic embryogenesis
- Virus elimination
- *In vitro* grafting
- *In vitro* gene banks, stock plant banks

*Corresponding author. E-mail: bunmiajisafe@yahoo.com.

- Somatic variation
- Managing ‘natural’ variation
- Induced mutation
-*In vitro* screening and selection
- Anther or microspore culture-production of haploids
- leading to double haploids
- Protoplast culture - somatic fusion
- DNA transformation systems
- Recovery of regenerants from transformed cells
- Cell culture
- Biosynthesis in bioreactors (production of secondary metabolites)

The greatest value of these technologies lies not so much in their application to mass clonal propagation but rather in their role underpinning development and application in plant improvement, molecular biology and bioprocessing, as well as their importance in research. The applications of plant tissue culture go well beyond the bounds of agriculture and horticulture. It has found application in environmental remediation and industrial processing. With rapid population growth, the total acreages of fruits, vegetables and various ornamental plants have not been able to meet the demands of the people in developing countries. Plant tissue culture techniques, an under-utilized tool in Nigeria can be extensively applied to increase horticultural crop production. However, for the purpose of this paper we focus on reviewing the range and scope of applications of plant tissue culture to agriculture and horticulture and highlight current or potential areas for further research and development.

MICROPROPAGATION

Plant tissue culture, also called micropropagation, is a practice used to propagate plants under sterile conditions or in a controlled environment, often to produce clones of a plant. In these processes, tissues or cells, either as suspensions or as solids is maintained under conditions conducive for their growth and multiplication. These conditions include proper temperature, proper gaseous and liquid environment and proper supply of nutrient. Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, or (less commonly) roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones (Vidyasagar, 2006). Tissue culturing, as applied to plants, is presently viewed as an expensive method. Although micropropagation represents one of the few means by which much forestry, plantation and other difficult-to-root species can be clonally reproduced, the high cost of tissue culture techniques has prevented broader application in the marketplace. Consequently, the appearance of clonal forests, fields and crops has not materialized.

Micropropagation allows the production of large numbers of plants from small pieces of the stock plant in relatively short periods of time. Depending on the species in question, the original tissue piece may be taken from shoot tip, leaf, lateral bud, stem or root tissue. In some cases, the original plant is not destroyed in the process - a factor of considerable importance to the owner of a rare or unusual plant. Once the plant is placed in tissue culture medium, proliferation of lateral buds and adventitious shoots or the differentiation of shoots directly from callus, results in tremendous increases in the number of shoots available for rooting. Rooted "microcuttings" or "plantlets" of many species have been established in production situations and have been successfully grown on either in containers or in field plantings. The two most important lessons learned from these trials are that this methodology is a means of accelerated asexual propagation and that plants produced by these techniques respond similarly to any own-rooted vegetatively propagated plant. Since plant tissue culture is a very labour intensive process, this would be an important factor in determining which plants would be commercially viable to propagate in a laboratory.

Advantages of micropropagation

Micropropagation offers several distinct advantages not possible with conventional propagation techniques.

i) Rapid multiplication of genetically uniform plants (clones) that possess desirable traits. A single explant can be multiplied into several thousand plants in a very short time. Once established, actively dividing cultures are a continuous source of microcuttings which can result in plant production under greenhouse conditions without seasonal interruption.
ii) The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds.
iii) The regeneration of whole plants from plant cells that have been genetically modified. Using methods of micropropagation, the nurseryman can rapidly introduce selected superior clones of ornamental plants in sufficient quantities to have an impact on the landscape plant market.
iv) The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests and pathogens.
v) The production of plants from seeds that otherwise have very low chances of germinating and growing, e.g. orchids and nepenthes.
vi) To clean particular plant of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture.

Applications of micropropagation

Plant tissue culture is used widely in plant science; it also

has a number of commercial applications. These include:

i) Screening cells rather than plants for advantageous characters, e.g. herbicide resistance/tolerance.
ii) Large-scale growth of plant cells in liquid culture inside bioreactors as a source of secondary products, like recombinant proteins used as biopharmaceuticals.
iii) To cross distantly related species by protoplast fusion and regeneration of the novel hybrid.
iv) Embryo rescue (the resulting embryo as a result of cross-pollination which would otherwise normally die is cultured in a medium to rescue it).
v) For production of doubled monoploid plants from haploid cultures to achieve homozygous lines more rapidly in breeding programmes, usually by treatment with colchicine which causes doubling of the chromosome number.
vi) As a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants.
vi) *In vitro* conservation of germplasm. This technique is mainly used to conserve plant which do not produce seeds or which have recalcitrant seeds which cannot be stored under normal storage conditions in seed gene banks. Hence, vegetatively propagated crops such as root and tubers, ornamentals, medicinal plants and many other tropical fruits have to be conserved using *in vitro* methods.

Micropropagation techniques

Micropropagation is a simple concept. The basic protocols were well established by the 1960s and a whole research field and industry grew based on the ubiquitous MS medium (Murashige and Skoog, 1962) and the numerous modifications that have followed. However, in reality, these protocols have been far less than universally successful. Many species and cultivars have not responded to existing protocols. Too often the protocols published by researchers for particular species are not reproducible by other laboratories or do not stand up under sustained production. This is not necessarily the fault of the original researchers, but rather indicates that we have not been taking into account all the critical factors involved in a commercially viable system. This situation has caused some researchers to revisit the basic principles.

Modern plant tissue culture is performed under aseptic conditions under filtered air. Living plant materials from the environment are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms, so surface sterilization of starting materials (explants) in chemical solutions (usually alcohol or bleach) is required. Mercuric chloride is seldom used as a plant sterilant today, as it is dangerous to use and is difficult to dispose of. Explants are then 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. Solid and liquid media are generally composed of inorganic salts plus a few organic nutrients, vitamins and plant hormones. Solid media are prepared from liquid media with the addition of a gelling agent, usually purified agar. The composition of the medium, particularly the plant hormones and the nitrogen source (nitrate versus ammonium salts or amino acids) have profound effects on the morphology of the tissues that grow from the initial explant. For example, an excess of auxin will often result in a proliferation of roots, while an excess of cytokinin may yield shoots.

A balance of both auxin and cytokinin will often produce an unorganised growth of cells, or callus, but the morphology of the outgrowth will depend on the plant species as well as the medium composition. As cultures grow, pieces are typically sliced off and transferred to new media (subcultured) to allow for growth or to alter the morphology of the culture. The skill and experience of the tissue culturist are important in judging which pieces to culture and which to discard. As shoots emerge from a culture, they may be sliced off and rooted with auxin to produce plantlets which, when mature, can be transferred to potting soil for further growth in the greenhouse as normal plants. The procedure for micropropagation consists of 4 stages:

i) Culture initiation
ii) Bud multiplication
iii) Plantlet regeneration and
iv) Acclimatization (Hardening or weaning) in a green house.

FACTORS AFFECTING *IN VITRO* GROWTH

Choice of explant

The tissue which is obtained from the plant to culture is called an explant. Based on work with certain model systems, particularly tobacco, it has often been claimed that a totipotent explant can be grown from any part of the plant. In many species, explants of various organs vary in their rates of growth and regeneration, while some do not grow at all. The choice of explant material also determines if the plantlets developed via tissue culture are haploid or diploid. Also the risk of microbial contamination is increased with inappropriate explants. Thus it is very important that an appropriate choice of explant be made prior to tissue culture. The specific differences in the regeneration potential of different organs and explants have various explanations. The significant factors include differences in the stage of the cells in the cell cycle, the availability of or ability to transport endogenous growth regulators and the metabolic capabilities of the cells. The most commonly used tissue explants are the meristema-

tic ends of the plants like the stem tip, auxiliary bud tip and root tip. These tissues have high rates of cell division and either concentrate or produce required growth regulating substances including auxins and cytokinins.

Some explants, like the root tip, are hard to isolate and are contaminated with soil microflora that become problematic during the tissue culture process. Certain soil micro-flora can form tight associations with the root systems, or even grow within the root. Soil particles bound to roots are difficult to remove without injury to the roots that then allows microbial attack. These associated microfloras will generally overgrow the tissue culture medium before there is significant growth of plant tissue.

Aerial (above soil) explants are also rich in undesirable microflora. However, they are more easily removed from the explant by gentle rinsing and the remainder usually can be killed by surface sterilization. Most of the surface microflora does not form tight associations with the plant tissue. Such associations can usually be found by visual inspection as a mosaic, de-colorization or localized necrosis on the surface of the explant. An alternative for obtaining uncontaminated explants is to take explants from seedlings which are aseptically grown from surface-sterilized seeds. The hard surface of the seed is less permeable to penetration of harsh surface sterilizing agents, such as hypochlorite, so the acceptable conditions of sterilization used for seeds can be much more stringent than for vegetative tissues.

Explant size and thin section culture system

The induction of a desired morphogenic event in vegetative tissues by appropriate *in vitro* manipulations would probably be the most significant advancement in plant tissue culture. The success in achieving such directed morphogenic events are largely determined by the cultured tissue itself. Several explant-related factors appear to influence the organogenic potential of the cultured tissue (Benson, 2000). These include growth conditions, whole plant physiology and genotype of the source plant. In addition, a negative correlation between the explant size and the number of cells potentially available for organogenesis has also been recognised.

However, this observation did not receive much attention from researchers until the late 1990s (Lakshmanan et al., 1995, 1996). In an earlier study, Lakshmanan et al. (1995) have shown that the production of orchid protocorms *in vitro* can be substantially improved by manipulating the size of the explant alone. For example, the number of protocorms produced by thin transverse sections (0.6 mm thick) derived from a single shoot tip (6-7 mm long) was 5 times greater than that produced by an intact shoot tip (6-7 mm long) cultured under identical conditions. A similar observation was also made recently in sugarcane. In this crop, leaf explants produced numerous plants (> 50 per explant) when the thickness of the explant was reduced to 1 to 2 mm (Lakshmanan et al., unpublished results). This finding clearly indicates that the explant size plays a key role in the expression of organogenic potential of the cultured tissue. This explant size-based difference in organogenic capacity has since been successfully utilised to develop thin section culture system, a novel approach in plant regeneration, of various commercial orchids (Lakshmanan et al., 1996), brassicas (Cheng et al., 2001) and more recently in wheat and sorghum (Lakshmanan et al., unpublished results).

The *in vitro* environment

In addition to work on aspects of *in vitro* biology such as autotrophy and hormone physiology such as auxin-regulated axillary growth (Reinhardt et al., 2000), some interesting areas of basic research that could improve our understanding and hence our ability to control *in vitro* plant regeneration and development remain under-explored. The development of recirculating liquid culture systems will make it feasible to monitor and continuously regulate the medium composition. We need to know more about the dynamics of mineral nutrition *in vitro* (Williams, 1995). Light quality has often been overlooked as a potentially important environmental factor, as it has been shown to affect the direction of plant morphogenesis *in vitro* (Morini et al., 2000) and the switch between gametophytic and sporophytic pathways. Tissue culture often involves extensive cutting and stress injury of tissues. We know that such stress causes programmed physiological changes in plants (Leon et al., 2001).

CURRENT DEVELOPMENTS (NEW CULTURE SYSTEMS)

Bioreactors

It is a well-established fact that most plants in culture grow better in liquid than on solid media. To further enhance the productivity of liquid culture systems, several innovative approaches were adapted depending on the final product desired and the species investigated (Aitken-Christie et al., 1995). Bioreactors for plant culture are the most prominent being adapted for a number of species. Since Murashige (1974) introduced the basic micropropagation plan, the application of bioreactors is one of the major developments that have occurred in the plant tissue culture industry. Compared to traditional tissue culture techniques, bioreactor systems offer several advantages; they are time and labour-saving, relatively easy to scale-up, allow enhanced growth and multiplication (e.g. by forced aeration) and improved nutrient availability due to the use of liquid medium. Several new strategies were adapted to develop bioreactors suitable for various plant species and their specific requirements (Aitken-Christie et al., 1995; Paek et al., 2001). The prin-

cipal systems are

1) Aeration-agitation bioreactor
2) Spin filter bioreactor
3) Gaseous phase bioreactor
4) Rotating drum bioreactor
5) Air-driven bioreactor (Lee, 2004).

These basic systems have already been used for the mass production of over 80 crops (Takayama, 1991) and are now being evaluated for production of several other plant species (Paek et al., 2001). As a plant production technique, bioreactors are far superior to traditional *in vitro* methods for all the species thus far tested. It is worth noting that with bioreactors, even the difficult-to-propagate woody and tree species can be produced relatively easily at high frequency.

For instance, an efficient, somatic embryo-based mass propagation system for the recalcitrant species *Coffea arabica* was recently developed using a bioreactor (Etienne-Barry et al., 1999). The normal and uniform development of coffee embryos achieved with the use of a bioreactor allowed direct sowing of embryos in the field, resulting in rapid crop establishment. In brief, bioreactors have the potential to improve product quality and substantially reduce the cost of micropropagation, but further development of technology is required to realize any commercial benefit from this system.

In Vitro mycorrhization

Traditionally, aseptic conditions were considered essential for plant tissue culture systems. More recently, attention has turned to the possible beneficial effects of microorganisms in *in vitro* plant cultures. For example, the root endophyte *Piriformospora indica* promotes explant hardening (Sahay and Varma, 1999); *Psuedomonas* spp. can reduce hyperhydricity (Bela et al., 1998) and *Bacillus pumilus, Alcaligenes faecalis* and *Psuedomonas* spp. improve shoot multiplication (Monier et al., 1998). Mycorrhization in micropropagation, particularly the use of arbuscular mycorrhizal fungi (AMF), is now gaining momentum due to a demonstrated positive impact on post-transplant performance of *in vitro* grown plants (Lovato et al., 1996; Rai, 2001). Improved nutrient uptake, water relations, aeration, soil pH balance (Sylvia, 1998) and their potential use as bioregulators (Lovato et al., 1996) have recently heightened research interest in AMF, contributing to the development of effective AMF production methods, mycorrhization of *in vitro* plants and screening for efficient AMF strains. The potential of different AMFs for application in commercial micro-propagation Industries can now be tested using an array of tools.

THE GENETIC BASIS OF *IN VITRO* MORPHOGENESIS

Like many other variables, genetic factors have long been identified as major determinants of *in vitro* growth responses of cultured plant tissues (Dunwell, 1981). In the 1980s, efforts were directed to unravel the nature of genetic control of plant tissue culture responses. While these studies have shown that additive gene effects appear to predominate, maternal and paternal genetic effects have also been implicated (Willman et al., 1989). The traits linked to tissue culture responses were highly heritable (Koornneef et al., 1987) and attempts to improve culture response through backcrossing have been successful (Koornneef et al., 1987). Some of the breeding programmes aimed at determining gene number concluded that relatively few genes are involved in controlling tissue culture responses (Koornneef et al., 1987; Willman et al., 1989). Rapid advances in molecular marker and recombinant DNA technology and the availability of well-defined mutant lines of model systems provide a major impetus to understand the genetic basis of *in vitro* morphogenesis.

Genetic factors controlling tissue culture responses have been identified in rice (Takeuchi et al., 2000), barley (Komatsuda et al., 1995), maize (Armstrong et al., 1992), *Arabidopsis* (Cary et al., 2001) and tomato (Bertram and Lercari, 2000). A very interesting study by Armstrong et al. (1992) determined the chromosome locations controlling somatic embryogenesis in the highly regenerative maize inbred line A188 and these loci were successfully introgressed into a 249 elite, recalcitrant line. The introgressed line showed a significant increase in somatic embryogenesis (nearly 90% of the explants produced somatic embryos compared to about 45% obtained with the parental recalcitrant line).

It is believed that these chromosomal locations could promote somatic embryo initiation and plant regeneration in other recalcitrant inbred lines as well. Analysis of the results reported so far in this area of research suggests that different genetic elements could be controlling shoot organogenesis and somatic embryogenesis in different species (Armstrong et al., 1992; Takeuchi et al., 2000), but at present it is evident that at least one dominant gene is associated with shoot regeneration in rice (Takeuchi et al., 2000). Also emerging is the information related to genes specifically involved in the perception of hormonal signals responsible for the induction of organogenesis (Cary et al., 2001) and those controlling the light-dependent acquisition of competence for shoot regeneration in vegetative tissues (Bertram and Lercari, 2000). Despite the exciting observations made in the recent past, this emerging field is still in its infancy as little is currently known about the fine details of the genetic networks regulating *in vitro* morphogenesis.

CHALLENGES IN MICROPROPAGATION

Somaclonal variation

Somaclonal variation has perhaps been the greatest

threat to the widespread application of micropropagation and other tissue culture techniques to agricultural crops (Peschke and Phillips, 1992). While in the early days of tissue culture application it was assumed that the plants produced would be clonal (that is, genetically uniform), it is now well recognised that this is not always the case. 'Off-types' are often produced which have been shown to be genetic mutants arising during the mitotic events in the tissue culture process, but are heritable mutations passed on to subsequent sexual reproduction cycles (Sahijram et al., 2003).

However, not all the variation arising from micropropagation is stable and heritable. Some variation arises from extra-nuclear DNA (epigenetic) and is therefore not uniformly inherited. Somaclonal variation must also be distinguished from phenotypic or physiological variation between explants, most commonly expressed in explant size or vigour. Such aberrations are not heritable, although they may have long lasting effects on the affected plants. It is generally thought that somatic mutation is induced by the presence of growth regulators in the culture medium and that the risk of variation increases with the extent of tissue differentiation involved in the culture system. Thus, somaclonal variation can be minimised by using 'simple' micropropagation, based on stimulation of existing axillary buds to grow-out rather than involving adventitious bud formation and or an intervening callus stage. Any commercial tissue culture propagation system must provide a means of screening for the occurrence of somaclonal variants.

This may be relatively easy where there is a distinct difference in the appearance of affected explants, but it is a much greater problem when the effect is physiological or does not appear until much later in the plant's growth (e.g. effects on flowering). Gene technology now offers powerful tools for this screening process and can enable detection of off-types early in the production cycle (Sahijram et al., 2003). However, some somaclonal variants have proved to be of agronomic and commercial importance and in a limited number of cases have been released as new cultivars. The potential improvements reported include enhanced resistance to fungal, bacterial and viral diseases, improved insect and nematode resistance, enhanced economic yield, improved drought, chilling, salinity and aluminum tolerance.

Culture decline – Habituation to hormones

Occasionally, after a prolonged period of continuous subculture, cultured tissues may spontaneously acquire the capacity to synthesize cytokinin and will therefore grow on a medium without added hormone. Habituation is a major limitation in commercial production systems resulting in the progressive decline in culture vigour and productivity with successive subculturing cycles. The concept of habituation to growth regulators and the need to transfer cultures to hormone-free media periodically has now been recognized. Many current protocols involve shorter periods on hormone-containing media with an intervening period on hormone-free media. Less attention has been paid to progressive changes in other chemical, physical and biological components of the culture system over time. However, there is a need to look more closely at the other constituents of media, including the mineral supply.

Conclusion

Plant tissue culture is now a well established technology which has made significant contributions to the propagation and improvement of agricultural crops in general. Greater contribution is envisaged from this technology in years to come, both in its own right and as an adjunct to the application of molecular biology. Understanding of the biological processes that permit the manipulation of *in vitro* morphogenesis and investigations on various physiological, biochemical and molecular aspects of plant hormones will greatly advance our knowledge and provide information that will help address the issues of *in vitro* recalcitrance or *in vitro* plant growth and development.

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