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Novel Micropropagation System: A Review

Pankaj Kumar Bhowmik and Toshiyuki Matsuiz
Department of Bioresource Production, Faculty of Agriculture,
Kagawa University, Miki-cho, Kagawa, 761-0795, Japan

Micropropagation, the true-to-type propagation of a selected genotype using *in vitro* culture techniques is originated from the pioneering research of previorus study who first attempted to culture isolated plant cells. As a concept rnicropropagation was first presented to the scientific community in 1960 by G.M. Morel producing virus-free Cymbkiiums. The necessary tools that made micropropagation a possibility, such as the development of media and an understanding of plant growth regulators, have been available only since the late 1950s and it was not until the early 1960s that a generalized culture medium was established. The actual establishment of commercial microipropagation as an industry became a reality during the 1970s and 1980s. But, in spite of the micropropagation industry being only 15 to 20 years old, significant progress has been made in the culture of plant tissues and cells *in vitro* and in the experimental manipulation of higher plant parts and cells as micro organisms (Jones and Sluis 1991; Zimmerman and Jones, 1991). Micropropagation consists of three types of vegetative propagation: (1) Somatic embryogenesis, in which structures are formed containing a shoot and root connected by a close vascular system (directly analogous to zygotic embryos in regards to histology, physiology and biochemistry); (2) adventitious shoot production, comprising *de novo mehstern* formation from callus tissue or directly from organized tissues such as epidermal or subepidermal cells; and (3) axillary shoot production, where axillary buds and meristerns give rise to shoots that are excised and used to produce additional such shoots. Numerous examples of successful applications have been reported for al menstem culture (b) organogenic micropropagation from undifferentiated tissues, cells or protoplasts (c) zygotic embryoculture (d) somatic embryogenesis and e) gametic embryogenesis. Hu and Wang (1983) have reviewed meristem, shoot tip and bud cultures. Meristem cultures have been employed to eliminate virus infection in same asexually propagated species. Meristem and bud cultures have been used commercially for multiplication of some high value genotypes. Zygotic embryo culture has been successfully employed to circumvent post-fertilization, cross-incompatibility during interspecific transfer of genes among related plant species for many years. Recently embryo rescue techniques have also proven useful in recovery of haploid plants that emerge from chromosome elimination in zygotic embryos following interspecific crosses. Regeneration of plants from callus, individual cells and protoplasts have involved organogenic or embryogenic differentiation. These techniques have been useful in providing both spontaneous (Scowcroft *et al*., 1987) and mutagen induced genetic variation isomaclonal/gametoclonal/protoclonal variance). Both embryogenic and organogenic differentiation have been involved among examples of plant regeneration following protoplast fussion, genetic transformation, anther culture and microspore culture. Ernbryagenesis has played a significant role in recovery of haploid plants from culture anthers or microspores. Thus, considerable progress in micropropagation technologies is evident and their applications in crop plants are diverse and significant. These technologies have been reviewed frequently during the past decade and the principles of *in vitro* culture are also presented in many good handbooks. So my objective is to present here only the novel micropropagation system emphasizing modern techniques of *in vitro* culture. I will review the application of novel micropropagation technologies for plant improvement along with the barriers, both scientific and business related, facing commercial micropropagation.

Novel Micropropagation Systems: The utility of any technology will ultimately be reflected in the relative value of information and products derived there from. That is why I will try in my discussion to explore utility of several micropropagation technologies through recent and potential applications as related to crop species.

Morphogenesls: The origin of form (morphogenesis) is an area of research with which tissue culture has long been associated. This is the area in which micropropagation made significant contributions both in terms of fundamental knowledge and application. Here morphogenesis is discussed briefly in two aspects.

Organogeneais: The classical findings of previours studies continue to be the guiding principles on *in vitro* organogenesis. *In vitro* organogenesis has been achieved in over 1000 plant species through empirical selection of the explant, the medium composition and control of the physical environment (Thorpe, 1990). Although progress is being made, the determinative events are not yet entirely known. in addition to traditional bulky explants such as cotyledons, hypocotyls and callus cell layers have been used In all systems examined, the organogenic process begins with changes in a single or small group of parenchyma cells, which then divide to produce a globular mass of cells or meristemoid. These cells can give rise to either a shoot or root primordium.

Embryogenesis: In contrast to organogenesis, which produces a unipolar shoot or root primordium, somatic embryogenesis gives rise to a bipolar structure with a root/shoot axis. Asexual embryogenesis has been reported in over 130 species, including cereals, grasses, legumes and conifers. The process can be divided into two major phases: the induction of cells with ernbryogenic competence and their subsequent development into embryos. Empirical manipulation of the explant, medium and culture environment has led to success in a process which is very plastic and may be structurally and/or cellularly different from zygotic embryogenesis (Ammirato, 1983). Carrot tissues have proven to be very useful in the study of somatic embryogenesis, but up to date most of the physiological and biochemical studies have dealt with embryo development rather than the whole process, However, the recent development of a method for selecting single cells, which form cell clusters and then somatic embryos in a relatively synchronous fashion (Normura and Komamine, 1985), is allowing for an in-depth examination of the entire process.

Clonal propagation: The use of tissue culture technology in vegetative propagation of plants has become the most widely used application of the technology in agriculture, horticulture and forestry. During the last 25 years it has become possible to regenerate plantlets from explants and/or callus from ornamental plants, food crops, vegetable and condiment plants, fruit and nut crops, medicinal plants and forest trees.

Ornamentals: Micropropagation of ornamentals has taken tissue culture out of the lab and into the commercial world. Today, for the ornamental industry, the use of tissue culture technology is as routine for some plants, as cuttings and other traditional methods are for others. All classes of ornamentals are propagated by tissue culture methods and many of the newer commercial labs have production capabilities of up to 200,000 *in vitro* plantlets per week. In ornamentals, more than in other sectors, chimeras are important. Different foliage plants with variegated leaves that are chimera are micropropagated (*Ficus benjamina* 'Golden King' and 'Golden Princes', *Ficus decora* 'Belgaplant', *Cordyline fruticosa* 'Kiwi' etc.). Probably orchids were the first horticultural plants to be propagated by tissue culture and G. M. Morel introduced the technique of meristem culture as a means of vegetative propagation. His method found almost immediate commercial use and a whole new market oriented plant propagation became available that placed orchids within the economic reach of the average person. Today the technique developed by Morel is used worldwide and few commercially important genera such as *Paphiopedilium* and *Phalaenopsis* which once remained fairly not feasible to micropropagate are now propagated.

Field and vegetable crops: Micropropagation protocols exist for large numbers of field and vegetable crops. Though once many agronomic crops have been difficult to manipulate *in vitro* and regeneration has been problematic, currently progress in regeneration has been significant, such that most crops can now be regenerated. Of the major field crops, reliable and efficient methods for the regeneration of cereals and other grain species have been available since 1980. This has been made possible by using immature embryos to produce stable regenerable embryogenic callus. The exception is sugarcane, traditionally propagated vegetatively and now micropropagated at least for some applications of seedcane field establishment. For other agronomic crops (as well as for sugarcane artificial seeds), the problems are production of thrifty, high-quality somatic embryos, control of somaclonal variation, development of an artificial endosperm (for albuminous species) and optimization of an efficient delivery system. Somaclonal variation can likely be controlled during the callus stage, at least partially by slowing and directing cell growth. initial observations with growing alfalfa callus on a high potassium citrate medium have shown marked reduction in variation of subsequent regenerated plants. For agronomic crops, micropropagation is currently commercial only for sugarcane. Micropropagation for other crops will likely be for development and production of hybrids, with alfalfa, cotton, rice, soybean and sugarbeet being of principal interest. Vegetable crop species have been used extensively in tissue culture research. Notable examples include carrot cell culture and embryogenesis research and potato protoplast manipulation and regeneration studies. Many reviews have elucidated the application of cell, tissue and organ culture techniques to vegetable crop species. Specifically, *in vitro* techniques to multiply plants by embryogenesis, organogenesis and non-adventitious shoot proliferation are reported in the literature with many vegetable species.

Fruit, plantation and forest trees: Protocols for the large-scale mass clonal propagation of several members of this heterogeneous group currently exist. Initial uses were for small (soft) fruit crops such as strawberry and raspberry and for rootstocks for several tree fruits, especially peach. As methods were developed for more crops and as experience was gained with micropropagated plants, shifts in patterns of usage have occurred. For both apple and pears, clonal propagation of root stocks and scion cultivar utilizes shoot tips. Several tropical fruit trees can be regenerated via somatic embryogenesis using nucellar tissue or by organogenesis using shoot tips and axillary buds. However, only banana is being exploited commercially. *In vitro* propagation techniques have also been successfully developed for oil, date, coconut, some ornamental palms and peach palm. Micropropagation techniques have been developed several berry crops including thornless blackberry, raspberry, blueberry and grape. Grape can be regenerated from axillary shoots, adventitious budding and via somatic embryogenesis, but none of these methods as yet allow for mass clonal propagation. Successful micropropagation of forest trees is a relatively recent phenomenon. Plantlets can be produced via organogenesis and somatic embryogenesis in both hardwoods and softwoods and present, protocols exist for over 70 angiosperms and 30 gymnosperms. Several woody species such as poplars, wild cherry, eucalypts, redwood and radiate pine are now commercially micropropagated, while others such as sandalwood, birtch, teak and loblolly pine show promise.

Pathogen-free plants and germplasm storage: Although these two topics appear to be unrelated, a major use of pathogen-free plants is for germplasm storage.

Production of pathogen-free plants: Crop plants especially those propagated vegetatively, are generally infected with pathogens. Plants infected with bacteria and fungi often respond to treatments with bactericidal and fungicidal agents, but there is no commercially available treatment to cure virus-infected plants. Meristem tip culture has been very useful in the elimination of virus from valuable genetic stocks normally multiplied through vegetative cuttings or tubers. The success of Morel and Martin in eliminating virus from infected dahlia plants in 1952 and from potato plants in 1955 stimulated stem tip culture in many horticultural species. Once isolated, virus-free clones can be maintained *in vitro* to provide disease-free stocks for multiplication through conventional or micropropagation (Hu and Wang, 1983).

Germplasm storage: Traditionally, germplasm has been maintained in the form of seeds, but this method has several limitations. However, the ability to regenerate whole plants from somatic and gametic cells and shoot apices is leading to their use for storage. Three common *in vitro* approaches have been used, namely growth retarding compounds, low temperature and cryopreservation.
A wide variety of growth retarding chemicals, including maleic hydrazide, 5995 and ABA have been used to reduce the growth rate of *in vitro* plantlets, so as to increase the time between subcultures. Germplasm can also be stored in cultures at non-freezing low temperatures (1-9°C). At these temperatures the aging of the plant material is slowed down, but not completely stopped. Consequently, subculture of the plant material is infrequent. In contrast to the above methods, most effort during the last 15 years have entered around

cryopreservation, where the plant material, after treatment with a cryoprotectant, is frozen and stored at the temperature of liquid nitrogen, More than 100 species have been cryopreserved at present including both monocots and dicots as well as conifers.

Plant modification and Improvement: *In vitro* methods are being used increasingly as an adjunct to traditional breeding methods for the modification and improvement of plants.

***In vitro* fertilization and embryo culture:** The technique of controlled *in vitro* pollination on the stigma, placenta or ovule can be used in several ways. These include the production of interspecific and intergeneric hybrids, overcoming sexual self-incompatibility and the induction of haploid plants. Embryo, ovary and ovule culture have been used in overcoming embryo inviability, monoploid production in barley and in overcoming seed dormancy and related problems. Embryo abortion is a common problem in breeding programs, due to failure of endosperm development. By aseptically culturing the embryo (or in some cases the ovary), this problem can be overcome. Interspecific and intergeneric hybrids of many important crops have been obtained by this embryo rescue approach. One novel use of embryo culture has been in the production of monoploids and double monoploids in barley. The causes of seed dormancy are varied, but excision of the embryo is often enough to allow germination, as in the case of the immature embryos of orchids, which led to the first commercial application of tissue culture technology. Embryo culture has also proven useful in reducing the breeding cycles of new varieties, in cases where long dormancy and/or slow growth of the seedlings resulted in long breeding seasons.

Haploidy: Natural haploidy has been known for a long time, but it was the studies on *in vitro* induction of androgenesis or the direct production of haploid embryos from anthers in the early 1960s, that led to renewed interest in this phenomenon. Haploids may be used to facilitate the detection of mutations and recovery of unique recombinants, since there is no masking of recessive alleles (Bajaj, 1983). The requirements for successful androgenesis or gynogenesis must be determined empirically. Direct regeneration, rather via a haploid callus is the usual route, but only a low percentage of the explants respond positively. At present, much more success has been achieved with androgenesis than gynogenesis, perhaps due to the earlier success with the former. Nevertheless, the latter has allowed for haploid production in certain genera for which anther culture is not an effective technique. Androgenesis has been reported in some 171 species, of which many are important crop plants (Hu and Zeng, 1984) and gynogenesis in 15 species. Androgenic haploids have been integrated into breeding programs as homozygous diploid lines. In china, this method has led to the development of new varieties of rice, tobacco and winter wheat and new breeding lines of maize and sugarcane (Hu and Zeng, 1984).

Somaclonal variation: In addition to the variants/mutants obtained as a result of the application of a selective agent in the presence or absence of a mutagen, many variants have been obtained through the tissue culture cycle itself. These somaclonal variants, which are dependent on the natural variation obtained in a population of cells, may be genetic or epigenetic and are usually observed in the regenerated plantlets. Many of the changes observed in *in vitro*

regenerated plants have potential agricultural and horticultural significance. These include alterations in plant pigmentation, seed yield, plant vigor and size, leaf and flower morphology, constituents of essential oils, fruit solids and disease tolerance or resistance. Such variations have been observed in many crops including wheat and triticale, rice, oats and maize, sugarcane, tobacco, tomato, potato and celery. The above types of variation obtained from somatic cells and protoplasts can also be obtained from gametic tissue. One of the major potential benefits of somaclonal variation is the creation of additional genetic variability in co-adapted, agronomically useful cultivars, without the need to resort to hybridization. This method could be valuable if *in vitro* selection is possible, or if rapid plant screening methods are available.

Use of protoplasts: Plant protoplasts can be routinely produced from many species using leaf mesophyil cells and cell suspensions and mixtures of cell wall degrading enzymes. These protoplasts have been used in fundamental as well as applied studies. The number of species that can be regenerated from protoplasts is steadily increasing.

Protoplast fusion: This has often been suggested as a means of developing unique hybrid plants, which can not be achieved via conventional sexual hybridization. While any two plant cells can be fused by chemical and physical means, production of unique somatic hybrid plants is limited by the ability to regenerate plants from isolated plant protoplasts. Most success has been limited to model plants from the genera *Nicotiana, Petunia, Datum* and *Hyoseyamus.* Only recently has some success been achieved in the Gramineae but none with grain legumes, although regeneration from protoplasts of several forage legumes has been achieved. Protoplast fusion can be used to produce unique nuclearcytoplasrnic combinations and to transfer cytoplasmically controlled male sterility between breeding lines. To date this has been achieved in *Nicotiana, Petunia* and *Brassica napus.* Populations of regenerated plants arising from protoplast fusion contain more variability than comparable populations of plants produced by sexual hybridization. Much of the research carried on to date has been directed towards the production of novel hybrids, not breeding line development. To make the technology more valuable for the production of new crop varieties emphasis must be placed in this area.

Vector-independent gene transfer: Genetic modification of plants is also being considered through uptake of DNA and organelles and single cell uptake in protoplasts. Genetic transformation through DNA uptake requires DNA from one source to be taken up, incorporated into the recipient cell in a stable form and that the genetic information encoded in the foreign DNA is expressed as new stable characteristics of the most recent developments for vector less transfer is the use of the particle gun or high velocity microprojectile technology. Here DNA is shot through the cell wall and into the cytoplasm on the surface of small metal particles that have been accelerated to speed of several hundred meters per second (Klein *et al.*, 1987). In this method stably transformed tobacco and soybean plants have been produced without going through protoplasts.

Vector-mediated gene transfer: The use of *Agrobecterium* as a tool for gene transfer has progressed very rapidly since the

first published report of stable transformation. Although the early transformation experiments utilized plant protoplasts, regenerable organ such as leaves, stems and roots have subsequently used with many dicot plants (Grasser and Fraley, 1989). In addition to vectors based on *Agrobacterium tumefaciens,* others have used *Agrobacterium rhizogenes* plasmids. Recent progress in this area has been so rapid that genetically engineered soybean, cotton, rice, corn, oilseed, rape, sugar beet, tomato and alfalfa crops are expected to enter the market place before the end of this decade.

Scientific barriers: Micropropagation remains a popular research subject and since it was first used as an index word in *Agricola* in 1976 more than 2200 entries have been listed in this category. Continued research is definitely needed to increase the speed with which some plants can be produced giving a superior quality product. There is often inadequate research in the development of protocols for proliferation, rooting, acclimation and re-establishment. Transplanting problems still exist. The ability to successfully take a plant, especially woody plants, through the four stages of micropropagation and consistently produce healthy, saleable plants still remains a challenge and requires coordinated planning. Testing for the presence of pathogens unfortunately is not a routine procedure and many laboratories produce pathogen-free plants by coincidence rather than by design. There is a great need for increased automation, as the majority of the costs associated with micropropagation are related to hand labor (Zimmerman and Jones,1991). Research funding, both private and public, needs to be increased if scientific barriers are to be resolved in a timely manner. The success of micropropagation is clearly dependent on maintaining the genetic integrity of a propagule to guarantee clonal identity. Practices that do not guarantee the maintenance of clonal identity, e.g., regenerative process, need to be avoided until such time that the causes of somaclonal variation are fully understood and controllable.

Business-related barriers: In the early years, micropropagation firms sold to finishing nurseries and expected nursery operators to educate themselves as to the handling and marketing of quality micropropagated plants (Jones and Sluis, 1991; De Metsenaere, 1991). This is changing. Micropropagation firms now a days directly alligned with a nursery to better ensure the high quality of the finished plants. Successful micropropagation requires a strategy to produce a large number of quality plants using very labour-itensive protocols based on a highly trained labour force. But the ability to produce a large number of high-quality plants does not guarantee success (Jones and Sluis,1991). Micropropagation must compete with conventional propagation, which often produces at a lower cost. Finally, it is also sensitive to prospects/constraints of general agriculture and horticulture (De Metsenaere, 1991). Micropropagation is extremely labor-intensive with nearly half of the costs attributable to labor. Research and development accounts for only very little. Cost reductions have been difficult because the cost for labor is directly associated with the transfer and maintenance of culture. One solution is to move to lower-cost labor sites, but there are problems with this strategy, including perishability of product, costs associated with long distance communication and unreliable electrical power sources. Part of the answer clearly lies in automation. This brief review of micropropagatoin system illustrates part of the ground work, since the field is vast and multi disciplined. The various systems discussed here are all being used in clonal propagation along with crop plant modification and improvement. Researchers in universities, government and industrial laboratories are all working in this area.

But, why is this necessary? The increased emphasis on sustainable agriculture and an increasing world population, coupled with the continued loss of prime agricultural land to housing and industry, signify that we will have to feed, clothe and. house more people than ever existed in the history of mankind. To do that we have two options. Either we have to cultivate new land or increase per unit crop yield. In real sense the first one is not possible. Then the alternative is to increase productivity and to do so micropropagation and other biotechnological systems are essential. Last of all, has micropropagation delivered all that it promised? Probably not, Micropropagation is a young industry with an excellent future and it will give the answer to many not all) problems associated with conventional propagation and other plant improvement techniques.

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