Development of Clones and Somaclones Involving Tissue Culture, Mycorrhiza and Synthetic Seed Technology

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Under current situation of rapidly increasing population, wastelands, and unemployment generation, it is necessary to produce the quality biomass at faster rate for various purposes, i.e., food, fibre, fuel, fertilizer, fodder, and agro-industries. In this regard, tissue culture has proved to be an efficient, eco-friendly, income generating, and technoeconomically viable technology which can be suitable for rural as well as semiurban areas. The present review deals with the work done in production of clones, somaclones along with mycorrhization, and synthetic seed technology for reclaiming the wastelands (mainly salt affected areas). Synthetic seed production is an appropriate technology for rural areas as it does not require sophisticated laboratories. Further, mycorrhization of micropropagated plantlets either involving tissue culture or synthetic seed technology hardens the saplings under field conditions.

Introduction

Plant cell/tissue culture is rapidly developing technology which holds promise of restructuring agricultural, horticultural, and forestry practices. The technique immensely reduces the labour and space requirements for producing new varieties of desired characteristics and can also markedly enhance propagation rates. Development of plant tissue culture is limited to improvement in techniques of protoplast, cell, tissue, and organ culture, followed by the success achieved in regenerating whole plant from cultured plant materials. Plant tissue culture is presently of great interest to molecular biologists, plant breeders, and industrialists. Tissue culture methods have been employed as important aids to conventional methods of plant improvement. It is particularly useful for multiplication of plants which are: slow growing; cross-pollinated, and show wide variations in the progeny; male sterile lines; newly produced varieties; for multiplication of virus free plants by meristem culture. It has also been used as a tool for the propagation of genetically manipulated superior clones, somaclones, in ex-situ conservation of valuable germplasm, and synthesis of many important secondary compounds. Clone propagation is a method by which identical plants of superior specimens can be obtained within a short span of time. Work on media standardization and conditions for clonal propagation of plants have been undertaken in several tissue culture laboratories. The present paper reviews the work conducted in the area of development of clones, salt tolerant somaclones, synthetic seed technology, and micropropagation along with mycorrhization.

1 Development of Clones (Micropropagation)

Micropropagation is defined as "the true-to-type propagation of a selected genotype involving regeneration of plants from organs, tissues, cells or protoplasts using in vitro culture techniques".

Ball1 has successfully raised transplantable whole plants of Lupinus and Tropaeolum by culturing their shoot meristem (tip). The potential of this work was soon realised by Morel2 for rapid propagation of orchids Cymbidium and Odontoglossum. The advantage of using this method was that about four million genetically identical plants could be obtained from a single bud. Murashige and Skoog3 have developed standard methods of propagation in vitro of species ranging from ferns to foliage, flower, and fruit plants. This new method of vegetative propagation has been exploited extensively in horticulture and the nursery industry for rapid clonal propagation of many
dicotyledons, monocotyledons, and gymnosperms worldover. The major advances in the mass propagation of woody plants have been made over the past 10 y.

In India also, over the past three decades, considerable work has been done in the application of tissue culture technology to horticulture, sylviculture, and agriculture. Micropropagation of elite forest tree genera such as *Santalum album*, *Eucalyptus, Tectona grandis*, and *Dalbergia latifolia*, even from tissues isolated from mature, 100-y-old trees and from the oil palm have been made at the Plant Bio-Technology Division of Bhabha Atomic Research Centre (BARC), Bombay; Indian Institute of Science (IISc), Bangalore; National Chemical Laboratory (NCL), Pune; Central Plantation Crop Research Institute (CPCRI), Kasaragod, Kerala, India. Significant contributions in the use of tissue culture methodologies for morphogenetic studies on a wide variety of test subjects was made by the Department of Botany, University of Delhi. The successful growth of ovary, ovule, and embryo parts in fruitset and seed development under *in vitro* conditions has been highlighted, as response to exogenous hormones.

Since 1980, micropropagation of several leguminous shrubby and timber trees has been receiving considerable attention. Also, clonal multiplication of many ornamental plants like orchids, roses and *chrysanthemum*, fruit crops such as apples, peaches, pears, strawberries, plums, and forest trees namely *Leucaena leucocephala*, *Prosopis cineraria*, *Dalbergia latifolia*, *Tectona grandis*, *Salix*, and *Fagus* has been undertaken by various researchers.

Recently, callus and plantlet regeneration from leaf explants in *Bauhinia sp.* from shoot tips of *Ficus elastica*; from shoot tips of cashew seedlings; from hypocotyl and cotyledon explants from various trees and in *in vitro* induction of flowering and fruiting in *Morus alba* was successfully performed. Various prospects of *in vitro* genetic manipulations like production of haploids, triploids, somaclonal variation, somatic hybridisation, and synthetic seeds of mulberry were described by Chand *et al.* High frequency bud break and multiple shoots were induced in nodal explants and apical shoots of various species of mulberry on Murashige and Skoog's (MS) medium supplemented with 6-benzylamino purine (1.0 mg/l) along with gibberellic acid (0.3 to 0.4 mg/l)\(^1\)\(^4\)\(^5\).

Mulberry plantlets were also successfully regenerated from callus cultures and interspecific protoplast fusion was observed.\(^6\)\(^7\)

2 Development of Salt- tolerant Somaclones

It is well known that genetic variations occur in undifferentiated cells, isolated protoplasts, calli, tissues and morphological traits of regenerated plants. The cause of variation is mostly attributed to changes in the chromosome number and structure. Cytological heterogeneity in cultures arises mainly due to such factors as:

(a) The expression of chromosomal mosaicism or genetic disorders in cells of the initial explants, and
(b) New irregularities brought about by culture conditions.

These variants selected in tissue culture have been referred to as callilones (from callus cultures)\(^8\) or protoclones (from protoplast cultures)\(^9\). Larkin and Scoeeford\(^10\) coined a general term Somaclonal variant for plant variants derived from any form of cell or tissue culture. The plant variants with salt resistant characteristics have been developed *in vitro* to reclaim salt affected wastelands. The diverse variation characteristic of somaclones highlights the fact that somaclonal variation may be an additional tool for crop improvement rather than an interesting scientific phenomenon.\(^11\)\(^12\) Somaclonal variations may be heritable as established in wheat and hence useful in generating progenies showing desired characteristics.\(^13\)

Plant tissue culture techniques have been used to develop salt tolerant somaclones either by directly exposing cells/cells/protoplasts/microspore/another or the explants to single salt(s) or their mixture in one step or gradually (stepwise) or indirectly by exposing to osmotic stress induced by mannitol or PEG (polyethylene glycol) or proline analogues.\(^14\) The potential advantages of tissue culture techniques for the production of salt tolerant plants are:

(i) Evaluation and selection of large number of genotypes in a small space in laborato-
(ii) Reduction of time between generations.
(iii) Control of environment and nutrient conditions.
(iv) Reduction of complications due to differences in morphology and stages of development as...
cells growing in culture are uniform in development.

(v) Generation of large variations with genotypes may be there during culture.

(vi) Selection of traits at cell level can be evaluated in regenerated plants and their progeny.

(vii) Use of isolated protoplast/cell/callus cultures to study the physiological and biochemical processes which regulate the salt stress tolerance.

The salt tolerant cell lines was first isolated from Capsicum annum by Dix and Street. Since then such cell lines have been isolated from more than 36 species of 28 genera belonging to 16 families. The variability generated in plant cells grown in culture can be examined for the desirable traits at two levels:

(a) Isolation of variants without cell selection – The callus is first regenerated into whole plants, which are then screened by conventional methods.

(b) Isolation of variants using cell selection – In this, millions of cells, each representing a potent plant are cultured in a single vessel exposed to conditions that retard or prevent the growth of normal cells while favouring the growth of desired variant cells.

Callus-based selection has been found to be inefficient and uncertain as all the cells in the callus piece are not uniformly exposed to the selective agent and this can result in stress avoidance due to cross-feeding between the cells in close contact with each other. The cell suspension cultures have yielded over 100-times more stable salt tolerance than callus pieces in Brassica juncea. But protocols for the establishment and regeneration of cell suspension cultures are available only in a limited number of plant species.

Several somaclones of different crop spp. viz. sugarcane, potato, tomato, geranium, cereals, grasses, and lucerne, are developed in India but so far no useful variant of tree species, particularly for salty areas has been developed. Salt tolerant cell lines of Pennisetum purpureum, an important perennial forage grass; have been successfully regenerated into plants. Salt tolerant cells in some of the plant species have been isolated by selecting the cells which accumulate higher levels of osmoregulatory compounds, e.g., proline quaternary ammonium compounds, polyols, or by selecting for tolerance to osmotic stress component of salinity. At high salt concentrations (1.2-2 per cent) maize callus showed significant increase in total polyamine content, especially caused by rise in putrescine. In sugarbeet callus cultures, the salinity tolerance of the normal callus is accompanied by the accumulation of proline under hypersaline conditions (274 mM). Similar effects of salt (NaCl) with ten-fold increase in proline in alfalfa cell lines, two to eight-fold increase in ornithine decarboxylase activity in roots and putrescine contents in leaves of Vigna radiata was observed. On the contrary, the stress treatments of 100 and 200 mM NaCl decreased the level of polyamines for the cultivated and wild tomato species. Salt stress increased the levels of diamine and polyamine in varying degrees among nine rice cultivars investigated by Krishnamurthy and Bhagwat. Many amino acids, including arginine, asparagine and serine were found to accumulate in both the sink and source tissues of Coleus blumei during salinity stress. In spite of this, NaCl induced an increase in ethylene synthesis and 1-aminocyclopropane-1-carboxylic acid (ACC) content in leaves of rice with exogenous putrescine application. Rice and Cicer arietinum calli could be grown under inhibitory levels of NaCl only in the presence of osmoregulants and there is accumulation of proline implicated as an anti-stress organic molecule in salt adapted callus. NaCl altered contents of nucleic acids, protein, polyamines, and the activity of agmatine deiminase during germination and seedling growth of rice besides increasing leaf mortality prior to normal phase of senescence. In vitro selection of NaCl tolerant cell cultures in two varieties of Oryza sativa was described by Paul and Ghosh. The organogenetic calli of Lycopersicon esculentum with tolerance of NaCl up to 20g/l and plantlets from callus grown on 15g/l NaCl were obtained and inorganic ion content in tolerant calli and somaclones was studied.

3 Mycorrhization of Tissue Cultured Plantlets

The acclimatization of in vitro derived woody plantlets often leads to very high mortality rate due to desiccation and microbial infection. Several efforts have been made to protect this by mycorrhizing the micropropagated plantlets both in vitro and in vivo conditions as both ecto- as well as endomycorrhiza have great potential in nutrient uptake, water absorption, biocontrol of pathogens, synthesis of growth hormones, and establishment of plantlets in
adverse soil and climatic conditions. It was observed in situ and in vitro by Dixon et al. that introduction of mycorrhizal fungi to unfavourable saline conditions may improve early plant survival and growth.

(a) Role of Endomycorrhiza

In several greenhouse experiments, inoculation of micropropagated woody plantlets with several spp. of AM (Arbuscular mycorrhiza) fungi increased survival of the transplants from 80 to 100 per cent compared with uninoculated controls despite increasing biomass production and nutrients level. Tissue cultured plantlets of two Paulownia species were inoculated with Glomus epigeium and the distribution of several nutrient elements in these mycorrhizae was detected with X-ray microanalysis, spectra of which showed that arbuscules, vesicles and hyphae contained more P, S, Mg, and Ca than the uninoculated plants. Influence of AM on phosphate fertilizer efficiency in two tropical acid soils planted with micropropagated oil palm (Elaeis guineensis Jacq.) showed that AM inoculation increased the fertilizer utilization coefficient of plants 2.7 to 5.6-fold.

Inoculation with efficient AM fungi enhanced growth and mineral nutrition of five oil palm clones and suppressed the variability between them. Micropropagated banana plantlets growing in a sterilized growing medium (1:1 mixture of soil and sand, containing six ppm of NaHCO₃ extractable P) were inoculated with Glomus mosseae or G. monosporum or supplied with 33 ppm KH₂PO₄, fortnightly. Both AM inocula enhanced growth, P and N uptake and biomass production of host plants as compared to the control. Moreover, G. mosseae appeared to be more effective as a symbiont than G. monosporum, and this was probably due to a higher level of root cortex colonization and arbuscular development. Mycorrhizal plants developed a more economical and efficient root system with good nutrient absorption. It was also observed by Fortuna et al. that growths of mycorrhizal inoculated micropropagated plantlets of apple and plum were comparable to those fertilized with P. Hence, mycorrhizal inoculation can be used as a biotechnological tool to overcome blocked apical growth and reduce P inputs to micropropagated fruit trees. In spite of this, mycorrhiza also appeared to alter the carbohydrates status in stems and roots as promoting total soluble sugar and starch contents in micropropagated Pyrus and Prunus spp.

Inoculation with AM fungi increased total shoot length, stem growth, and biomass of the plants with a well developed mycorrhizal root system which also improved top soil fertility. Significant changes in root system morphology, acclimatisation and growth of rooted microcuttings was observed recently in micropropagated plantlets due to mycorrhizal symbiosis.

Mycorrhization of plantlets in India was mainly done by in vitro inoculation of AM fungi for proper hardening of developed plants. Recently, evidence was documented for the secretion of growth promoting metabolites like indole acetic acid (IAA) and kinetin by AM fungi along with associative growth and development of micropropagated plantlets. In vivo inoculation of endomycorrhizal spores in micropropagated plantlets of Populus deltoides resulted in proper hardening at field level. Similarly, inoculation of AM fungi on TC (tissue culture) plantlets of jackfruit showed proper establishment of these in field conditions. In a subsequent study on ex vitro inoculation of Leucaena leucocephala with Glomus fasciculatum, not only plantlets resulted in higher growth rate of root/shoot, number of leaves, number of nodules, and mycorrhizal colonisation but also only 20 per cent of these survived in soils lacking AM fungus.

Similarly, benefits of in vitro 'biotization' of plant tissue cultures with microbial inoculants including AM fungi was described recently by Nowak. For the first time, the axenic AM inoculum of Glomus epigaenum was produced on Trifolium pratense with the root organ culture techniques and applied to the test tube plantlets of Paulownia albibhoa. Chemical analysis of plantlets showed inoculated leaves with more N and P, same K, less C as compared to uninoculated controls. The work on the effect of phosphorus sources on endomycorrhizal infection of micropropagated bananas in vitro has also been reported.

Also, it has been shown by several workers that the mycorrhization of micropropagated plants at the rooting phase in the sterile tube is much laborious than the mycorrhization at the acclimatisation phase and does not have additional advantage and inoculation at the time of transplanting was much more effective in stimulating growth.
Endomycorrhiza has also been reported to act as bioprotector from diseases and pathogen attack, especially the nematode attack to micropropagated woody plants. Micropropagated pineapple plants of two varieties, Queen Tahiti and Smooth Cayene (clone CYO) were inoculated at transplanting stage from axenic conditions with an arbuscular mycorrhizal fungus to evaluate the importance of endomycorrhiza development for biological protection against Phytophthora cinnamomi. Growth and mineral nutrition of endomycorrhizal plants were not affected by pathogen in comparison to reduction in growth of control, however, symbiotic functioning was reduced by the highest concentration of inoculum of P. cinnamomi.

(b) Role of Ectomycorrhiza

Ectomycorrhiza like endomycorrhiza also has a growth enhancement effect over micropropagated plants while inoculating in vitro in vivo. The advantage of exploiting ectomycorrhiza in vitro is that not being obligate, it can be easily cultured.

Various species of ectomycorrhiza like Xerocomus badius, Paxillus involutus and Suillus lacteus have good potential to store N, P, K, Mg, Fe, Zn, Al, and Cd, and thus help in uptake and storage of macronutrients, ameliorate metal toxicity, and bioremediate the contaminated soils. Isolates of *Paxillus tinctorius*, *Suillus luteus*, and *Laetiporus sp.* were found to be highly tolerant to salts like NaCl and Na$_2$SO$_4$ in vitro conditions. *P. tinctorius* was found to be most effective in colonizing roots of *Castanea sativa* and mycorrhization of micropropagated plants increased survival and growth during weaning.

It was later observed by the Martins et al. that physiological parameters of micropropagated *Castanea sativa* was greatly influenced by *P. tinctorius* as it increased protein content and photosynthetic rates, decreased the respiratory rates, overall mycorrhiza improved plants physiological status, thereby enhancing the acclimatization process. It was also shown by Simoneau et al. while inoculating birch (Betula pendula) micropropagated plantlets with seven different isolates of the mycorrhizal fungus *Paxillus involutus* that specific polypeptides were synthesised during ectomycorrhiza formation. Partial sequencing of one clone have shown that it contained a portion of the gene for phenylalanine ammonialyase.

_Eucalyptus marginata_ shoots and *Pisolithus tinctorius* were co-cultured to obtain ectomycorrhizal formation, in vitro. Successful combinations resulted in formation of a mantle followed by a Hartig net and epidermal cell elongation. Genotype, maturity, and fungal specificity are key factors influencing successful ectomycorrhizal formation of _E. marginata_ by _P. tinctorius_, in vitro.

Pear shoot microcuttings inoculated with 1 ml/l *Hebeloma sinapizans* mycelium slurry on MS medium supplemented with various concentrations of IBA showed three-fold enhancement of rooting percentage in _H. sinapizans_ inoculated auxin free medium. Root number/plantlet was highest in inoculated media with or without IBA, but most roots were formed with IBA at 0.2 mg/l.

The rooting pattern of _Larix laricina_ hypocotyl cuttings under the influence of *Laccaria bicolor* was assessed by Stein and Fortin and compared with other plant growth regulators. The results revealed that the hypocotyls cultured with the fungus in the auxin supplemented media did not exhibit any callus tissue formation and numerous root primordia were initiated on the hypocotyl axis. Excised hypocotyls of _Pinus halepensis_ were cultivated in vitro with/without *Hebeloma hiemale* on rooting media unamended or supplemented with IAA or tryptophan. _H. hiemale_ strongly stimulated rooting on media supplemented with tryptophan.

On the contrary, when three ectomycorrhizal fungi, _Hebeloma crustuliniforme*, _Paxillus involutus*, and _Thelephora terrestris_ were inoculated in a peat/vermiculite mixture during the transition period of poplar plants from in vitro to glass house, survival of inoculated plants was found to be lower than that of uninoculated controls and lowest survival was observed in the presence of _T. terrestris_. But this fungus, however, gave the highest frequency of root infections with a significant increase in shoot height, doubling of shoot dry weight and an increase in N, P, and K contents. Micropropagated plantlets of chestnut were inoculated with _Boletus edulis_ and _B. reticulatus_ mycorrhizal fungi and their antagonistic effects towards Phytophthora (responsible for chestnut root rot) were studied by Chauvin and Saleses revealing antipathogen activity of ectomycorrhiza.
4 Synthetic Seed Technology

Synthetic or artificial seeds are the living seed-like structures derived from somatic embryos/shoot axillary or apical buds in vitro after encapsulation by a hydrogel. The synthetic seeds provide a potential method to combine the advantages of clonal propagation with the low cost, and high volume capabilities of seed production. These also include the rapid and large scale multiplication, minimal labour, and low cost propagation of seedless, hybrid, and many vegetatively propagated readily infected plants. This technology is useful for multiplying transgenic plants, somatic and cytoplasmic hybrids, sterile and unstable genotypes and also for cryopreservation of desirable elite genotypes. In addition, artificial seeds can be directly delivered to the field, thus eliminating transplantation and tissue hardening steps of micropropagation. The encapsulated embryos could also be packed with pesticides, plant growth regulators, fertilizers, nitrogen fixing bacteria, and even microscopic destroying worms.88,89

Redenbaugh et al.89 first discovered that hydrogels such as sodium alginate could be used to produce single embryo artificial seeds in alfalfa and celery. The most useful encapsulation system has been found to be the dripping of 2 per cent sodium alginate from a separator funnel into a 100 mM calcium nitrate solution. Since then, artificial seed production and subsequent plant development of many important crop plants like brinjal, carrot, Brassica, lettuce, sandalwood, rice, etc., has been demonstrated exploiting somatic embryos, axillary/adventitious buds, and shoot tips as in vitro propagules for encapsulation.91,92 For the production of artificial seeds in tree species, the recovery of plants (conversion) is crucial. Though difficulties in developing somatic embryogenesis systems in tree species are similar to those encountered in herbaceous species, tree species exhibit additional complexities. These are:

(i) Tissue culture dependent variabilities are high.
(ii) Genetic variation may be higher in tree species than cultivated herbaceous species.
(iii) Size of genome is quite large for many trees.
(iv) Mature plant material is to be collected from field.

In spite of these additional variables which increase the potential difficulty of developing repeatable somatic embryogenesis systems in tree species, there has been tremendous progress in initiating somatic embryogenesis in trees.93 Mangifera indica was the first tree species for which the viable artificial seeds have been produced.95 Afterwards synthetic seeds of mulberry,95,96 eucalyptus,97 and banana91 have been successfully produced. Following approach for genetic engineering of tree species with the help of artificial seed technology is recommended:

(i) Production of large scale embryogenic tissue from transformed (genetically engineered) cells or tissues.
(ii) Production of synchronous somatic embryos.
(iii) Maturation of somatic embryos.
(iv) Non-toxic encapsulation / coating as artificial seeds transformation and ‘progeny’ testing for low genetic and epigenetic variation, expression of engineered trait.
(v) Cryogenic storage of potential superior lines.
(vi) Greenhouse/nursery establishment, growth and transplanting into field or planting through direct delivery.

There are certain problems encountered in artificial seed production like:

(a) Various chemical and physical procedures are required to make embryo quiescent and viable for several months.
(b) Protection of artificial seeds is required from desiccation.
(c) Conversion from these seeds is often very low which may be due to improper development of embryo, or difficulties to arrest growth or because of difficulties in development of ‘artificial endosperm’ within the capsule.
(d) Protection of embryo against microorganisms is required by using fungicides and antibiotics. In later stages, however, inoculation with mycorrhiza may be beneficial.98

Conclusions

The development of clones (micropropagation), somaclones, mycorrhization of micropropagated plantlets and synthetic seed production are the efficient technologies for fast production of many disease-free, superior plants with desirable traits in a limited space for reclaiming wastelands. An integrated approach of these technologies is required to overcome the increasing demand of food-supply and other valuable needs.
In future, it is required to increase the development of clones/somaclones of important plant species and the screening, isolation, and multiplication of specific strains of mycorrhiza to protect these micropropagated plantlets under field conditions. Further, it is also desirable to enhance the output of somatic embryos per gram callus tissue, conversion frequency of embryos, preservation and protection of artificial seeds and encapsulation of other propagules to develop new synthetic seed coatings. The application of synthetic seeds should also be found in rapid delivery of tissue cultured developed clones and somaclones of different plant species.

These technologies would not only play vital role in reclamation and afforestation of wastelands, but also helped in meeting the gap between demand and supply of various biomass produce.

References


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