Review

Micropropagation as a tool for experimental breeding, crop production and crop improvement

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Biotechnology is the discipline which deals with the use of living organisms or their products. In this wide sense, also traditional Agriculture may be seen as a form of Biotechnology. Modern biotechnological techniques have rapidly expanded the horizons of plant Experimental breeding and crop improvement. Conventional Experimental breeding techniques rely on the random rearrangement of existing gene between two closely related parent plants. Now, genetic engineering has opened up new possibilities by allowing the transfer of individual known genes, even from completely unrelated organisms such as fungi or bacteria. In recent years, Biotechnology has developed rapidly as a practical means for accelerating success in Plants experimental breeding and improving economically important crops. The most important methods used to achieve these goals are: Mass propagation of scientific clones, Production of pathogens free plants, Clonal propagation of parental stock for hybrid seed production, Year round nursery production and Germplasm preservation. This paper reviews the importance of micro-propagation as a tool in biotechnology used for Experimental breeding, crop improvement and production

Keywords: micro-propagation, Experimental breeding, crop production, crop improvement

INTRODUCTION

Breeding is the art and science of changing and improving the heredity of an organism. The art of breeding lies in the ability of the breeder to observe in organisms differences which may have economic values. Before the breeders possessed the scientific knowledge, that is available today, they rely on their skills and judgment in selecting the superior type. Many breeders were quick observers, quick to recognize differences between organisms of the same species (variation) which could be used as basis for establishing new varieties.(Geneive, R.I. 1989).

Since 1900, Mendels laws of genetics provided the scientific basis for Plant breeding. As all traits of Plants are controlled by genes located on chromosomes, conventional plant breeding can be considered as the manipulation of the combination of chromosomes. In general, there are three main procedures to manipulate plant chromosome combination. First, Plants of a given Population which show desired traits can be selected and used for further breeding and cultivation, a process called (Pure line) selection. Second desired traits found in different plant lines can be combined together to obtain Plants which exhibit both traits simultaneously a method termed hybridization. Heterosis a phenomenon of increased vigor, is obtained by hybridization of inbred lines.

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Third polyploidy (increased number of chromosome set) can contribute to crop improvement. New genetic variability can be introduced through spontaneous or artificially induced mutations (Monti, 1997).

As breeders knowledge of genetics and related plant science progressed, breeding became less of an art and more of a science. No longer was it necessary for the breeders to rely so completely on his skill in finding changed variations with which to establish new varieties. It has now become possible to plan and create new types of organisms more or less at will. The breeder's scientific knowledge gave them the background to manipulate and direct the inheritance of an organism.

Experimental Breeding

This mode of Experimental breeding involves the crossing of genetically diverse lines of organisms, which leads to the production of different combination of alleles in one line .For example parental lines are crossed, producing an F1 which is then allowed to undergo random mating to produce offspring that have pure breeding genotype (i.e AA, bb, cc or DD).This type of experimental breeding is the beginning of new plants.

Micropropagation

Tissue culture is an aseptic laboratory procedure that requires unique facilities and special skills. The technique of tissue culture is the culture and maintenance of plant cells, organs or tissues in sterile nutritionally and environmentally supportive condition outside living system. In 1938 Schwann and Schleiden put forward the theory of 'totipotency' which states that cells are autonomic and in principle are capable of regenerating into a complete plant. This theory was the foundation of plant cell organ and tissue culture.

Haberlandt, (1902) made the first attempt to culture plant cell and tissue but failed. He attempted the culture of single cells isolated from the palisade tissue of leaves, pith parenchyma, epidermis and epidermal hairs of various plants, but obtained no cell division. However between 1907 and 1909, Harrison, Burrows and Carrel succeeded in culturing animal and human tissue in vitro. Although earlier workers had achieved in vitro culture of orchid seeds (seedlings) embryos and plant organs, in 1960, cocking succeeded in obtaining the first real plant tissue culture. Following the discovery by White in 1937 demonstrated the importance of B vitamins of auxin (IAA) in the control of plant growth, other workers started to include these growth factors in their media, with increased growth responses.

In 1939, progress in tissue culture was slow. Development consisted merely in report of establishment of tissue cultures from many plant species. The studies of Camus in 1949 on the induction of vascular differentiation resulting from grafting buds in callus tissue culture led to the important studies on factors controlling vascular tissue differentiation by Wetmore and Sovokin (1955), and Jeff and Northcote (1967). The work of Miller and Skoog (1953) on bud formation from cultured pith explants of tobacco led to the discovery of kinetin (a cytokinin) and the function of cytokining/auxin interaction.

In 1952, Steward initiated work on cultured carrot explant which for the first time involved analysis of culture growth in quantitative terms and was to lead to the wide use of coconut milk as a nutrient, and to the discovery of somatic embryogenesis (Steward, 1958; Reinert, 1958). The potential of single cells to regenerate the entire plants through renewed embryogenesis was fully exploited and highlighted, thus the concept of 'totipotency'.

In 1959, Melchers and Bergman cultivated tissue derived from the haploid shoot of Antirrhimum majus. The tissue retained it's haploid state during several sub cultures but then, later increased in ploidy. Haploid tissues and suspension cultures are clearly of particular interest in mutation studies. Later on, Cosgrove, (2001) announced their discovery of organogenesis and embryogenesis from pollen cultured Datura anthers.

The release of protoplast from root tips cells using fungal cellulose in 0.6m sucrose was reported by Cocking (1960). Protoplast releases by cell wall degrading enzymes have now been prepared from many plant tissues, including suspension culture cells (Ericson and Johanson, 1969). Such cultivated protoplasts could reform their cell walls and divide in culture. Furthermore it was shown subsequently that macro – molecules can readily be introduced into protoplast and that protoplast fission can be promoted and controlled, leading to hybridization and transformation

Plant parts are cultured under controlled environment in small containers where all the nutrients required for growth are provided. Specific growth regulators are supplied to control growth and development.

There are a number of reasons to produce plants in tissue culture. These include:
i.   Natural products synthesis for such chemicals as natural flavors, colors, pharmaceuticals and even plastics.
ii.   Culture to produce haploid plants for the crop breeding
iii.   Crop improvement through biotechnology. This include gene transfer somaclonal variation and protoplast fusion.

Characteristics of Micropropagation

Micropropagation has become an important part of scientific breeding for many plants (Boxus. and Dwart,1980).The advantages of micro-propagation as a

propagation system have been reviewed so many times and can be summarized as

i.   Mass propagation of scientific clones
ii.   Production of pathogens free plants
iii.   Clonal propagation of parental stock for hybrid seed production.
iv.   Year round nursery production
v.   Germplasm preservation.

Mass Propagation of Specific Clones

The objective of mass propagation is to reproduce (either sexually or asexually) copies of an original parent. The controlled aspects of micropropagation permit the rapid propagation of individuals from a single plant. Multiplication rates can be very high, since plant in culture can theoretically be multiplied at an exponencial rate by consecutive subcuturing (e.g one month apart). Mass micropropagation is partially useful for the following:-

•   Plants whose natural rate of increase is relatively slow e.g herbaceous perennial hosta plant.
•   New cultivars requires getting to market in as short a time as possible.
•   Cultivars   whose   high   value   makes micropropagation a viable alternative to convensional methods.
•   Conservation of endangered species e.t.c

Production of Pathogen Free Plants

Production of propagation material free of fungal, bacterial, and systemic viruses and virus like pathogen has became an essential aspect of propagation (Hudson *et al.,* 2004). Micropropagation provides a method to rid a clone of external pathogens and can also provide a system in which plants are kept free of re infections until delivery.

Clonal Propagation of Parental Stocks for Hybrid and Seed Production

This procedure is used for marigolds asparagus, tomatoes, cucubits and broccoli.

Provision of Year Round Nursery Production

Most nursery operation are seasonal. Micropropagation has the potencial for continuous year round operations with production scheduled according to market demands. High volume production requires high volume distribution and the facilities to stock pile items.

Germplasm Preservation

The major method of germplasm preservation is seeds. However seed storage requires relatively large facilities with specialised storage conditions. Also seed storage is

not an option for recalcitrant seeds that do not tolerate dry storage conditions(Hudson, *et al* ,2004). For these reasons, preservation of vegetative tissue as explants is an attractive alternative.

Availability of Plant Germplasm for Use in Crop Improvement

Broad-based plant germplasm resource (PGR) are imperative for sound and successful crop improvement programs. Rich and diverse source also fuel many facets of plant research. For highly successful research and breeding activities, the genetic diversity of experimental materials needs to be sustained to minimize the vulnerability inherent in the growing of uniform and closely related cultivars over wide areas.(Stalker, and Murphy,1992).

A complete array of germplasm in a crop consist of
i.   Wild relatives, weed, races and landrace in the areas of diversity
ii.   Un-improved or purified cultivars used earlier in the major production areas or still used in minor areas.
iii.   Improved germplasm in commercial production and genetic testers from breeding programmes and genetic studies

Crop germplasm can be made available to the users(Agronomist, breeders, entomologist, genetics, plant pathologist, plant physiologist, crop ecologist soil scientist, production specialist, and growers) on a continuing basis of the interrelated facet of field conservation, multiplication, characterization, documentation and distribution are well handled by the germplasm workers (Stalker H.T and Murphy J.P
1992).

Disadvantages of Micropropagation

Micro propagation on a commercial scale has particular characteristics that may create problems that could limit use. These include:
i.   Expensive and sophisticated facilities, trained personnel and specialized techniques.
ii.   High cost of production result from expensive facilities and high labor inputs
iii.   Adequate storage facilities and growth chamber to stock pile productions.
iv.   Contamination or insect/microbial infestation can cause damage in a short time.
v.   Variability and production of off-types individuals can be a risk in the products emerging from micro-propagation

General Laboratory Facilities and Procedure

Facilities and Equipment

Facilities for aseptic procedures may be placed into three (3) categories as determined by their scope, size, sophistication and cost. These categories are:

i.   Research laboratories where precise work requires highly sophisticated equipment.
ii.   Large commercial propagation facilities where several millions of plants can be mass produced.
iii.   Limited facilities for small research laboratories.

  No matter what size, the facilities should include three basic components
i.   Preparation Area
ii.   Transfer Area
iii.   Growing Area

Preparation Area

The preparation area is essentially a kitchen with three basic functions.(a) glassware cleaning (b) preparation and sterilization of media and (c) storage of glassware and supplies. An efficient method of working is required either by hand or machine. Normally washing is followed by rinsing in distill de-ionized water. A sink with running water and electrical or glass outlets for heating are necessary, air or vacuum outlets are often useful. Table surface should be made of a material that can be cleaned easily.

   The following equipment items are used in the preparation area
i.   Refrigerator to store chemicals ,stock solutions and small batches of media
ii.   Scales or analytical balance, preparably top loading.
iii.   Autoclave capable of reading 120 degree centigrade. A house hold pressure coocker can be used to sterilize small batches of media
iv.   A PH meter, indicator paper can be used as a substitute in less precise work
v.   Gas or heating plate
vi.   Stirrer and mixing device
vii.   Filter for sterilizing non autoclavable ingredient(particularly in research laboratories).
viii.   Equipment to purify water. a glass still, an exchange reverse osmosis or a combination in small operations, distilled water may be purchased and stored in plastic containers.
ix.   A vacuum pump or an ultrasonic cleaner. These specific items can be used to decontaminate explants.
x.   A media dispenser valuable as a labor saving device.
xi.   Storage for flasks, bottles and other supplies.

Transfer Area

The transfer area is the place where explants are inserted in to culture; and where transfers of subcultures to fresh media take place. The key requirement is that the environment of the transfer area must be sterile and free from any contaminating organisms. Transfer is most conveniently done in an open sided laminar air flow hood, where filtered air is passed from the rear of the hood out ward on a positive pressure gradient. Air passes through a pre filter to remove dust. Then its drawn through a high efficiently particulate air (HEPA) filter that removes microbial spores. The hood is turned for at least 30 minutes before use. With such equipment the transfer may be carried out in a room with other activities.

Ultraviotet (UV) germicidal lamps can be used to sterilize the interior of the transfer chamber. These are turned on about two (2) hours prior to using the chamber but must be turned off during operation (Bridgen and Bartuk, 1987). The light should be directed inward not towards the workers face, because UV light may adversely affect the eyes.

Growing Area

Cultures should be grown in a separate lighted facility where both day length and light irradiance can be controlled and where specific temperature regimens can be provided. If different kinds of plants are to be propagated it is useful to have several rooms, each programme to meet the temperature and light needs of specific kinds of plants.

Containers for Growing Cultures

Test tubes, Erlenmeyer flasks, petri dishes and shell vials of various sizes are routinely used in research laboratories. They may be pyrex glass but are often less expensive soda glass and flamed during transfer operations. Various kinds of plastic containers are also available that are less expensive, less likely to break and often disposable.

   Various kinds of closures are necessary for the containers non absorbent cotton plugs have been used in research laboratories but are inconveniently used in large commercial operations. Metal or plastic covers or polyurethane or polypropylene plugs are more convenient. A second cover of aluminum foil or various plastic films is desirable for holding moisture and reducing infection but allowing exchange of Co_2, O_2 and ethylene.

Media Preparation

   A. *Ingredients*: Ingredients of the culture medium vary with the kind of plant and propagation stage at which one is

working. In general certain standard mixtures are used for most plants but exact formations may need to be established by testing. Media can be made from the Pure chemicals or purchased as commercial premixed culture media. Empirical trails may be necessary to test available combinations of ingredients when dealing with a new kind of plant. These ingredients can be grouped into categories of;

i.   In-organic salt
ii.   Organic compounds
iii. Complex natural ingredients
iv.   Innert supports.

i. In-organic salts: Inorganic salts provide the macro element (nitrogen, phosphorus, potttassium, calcium, copper, magnese, iodine, iron and zinc). These salt can be made in stock solution by group and stored in a refrigerator. A stock solution is a concentrated solution from which portion is removed to make the final medium. Stock solution save time because dry salt need only to be weighed once. The reason for preferring different stock solution is that certain kind of chemicals when mixed together will precipitate and not remain in solution. Stock solution should be stored in refrigerator (not frozen) and are good for several months cloudy stock solution should be discarded.

ii. Organic Compounds: Carbohydrate:- Sucrose at 2-4 percent is used for most cultures but concentration as high as 12 percent might be used in some cases as for young embryos. Glucose has some times been in use for monocots and selected dicots (like strawberry). Fructose, maltose and starch have been used occasionally. These materials are added at the time of making a culture.

Vitamins:-   Thiamin (0.1 to 0.5mg/l) is almost always considered. Essential and nicotinic acid (0.5mg/l) and pyridoxine (0.4mg/l) are usually added. Inositol at 100mg/l is beneficial in many cultures and is usually added rountinely. Other vitamins that are sometimes beneficial include pantothenic acid (0.1mg/l) and bioten (0.1mg/l). All of these substances are soluble in water and should be prepared as stock solutions, ready for dilution at 100 times the final concentration.

Horimones and Growth Regulators:- The two most important classes that are used to control organ and tissue development are the auxins and cytokinins. Gibberellins have some times been used to promote shoot elongation.

Auxins:- The natural auxin, indoleacatic acid (IAA) is usually more stable (Broome,1986) and include Neplithaleneacatic acid (NAA) (0.1 to 10mg/l), 3 indole-butyreic acid (IBA) (0.1-10mg/l) and 2, 4-Dichlorophenoxyacetic acid (2, - 4-D0 (0.05mg/l).

CYTOKININS:-   The compounds include N6-benzyladenine (BA), Kinetin, N6 isopentenyl – adenine (zip), and zeatin. They are used at a reabloe of 0.01-10mg/l. Thidiazurom and N-2 chloro4pyridyl, N-phenylurea (cppv)   have   cytokinin   activity   and   are

often used in combination with traditional cytokinin.

Gibberellins:- Gibberellic acid (GA_3) is sometimes used but must be filter sterilized. All of these materials should be prepared in advance and maintained as stock solutions in a refrigerator at near freezing temperature. Stock solutions of Auxins and other organic materials deteriorate with time.

Miscellaneous:- Citric acid (150 mg/l) or ascorbic acid (10mg/l) can be used as antibrowning agent (antioxidant). Malic acid (100mg/l) is added in some embryo culture media. These should be filter sterilized since autoclaving causes decomposition. These organic acids may be incorporated in medium or used in pre-washing steps. Fine grade activated charcoal preferably prewashed is used in some cases (0.1-1percent) to adsorb and counteract inhibiting substances (Brown, and Thorpe, 1984)

iii Complex Natural Ingredients:- Various materials of unknown composition have been used to establish cultures when known substances fail to do so. Protein hydrolysates from casein or other proteins are sometimes helpful, primarily for providing organic nitrogen and amino acids. Coconut milk has been used but must be filter sterilized for some uses. These substances are dissolved in water and added during preparation of the medium. Most of these materials are available from commercial sources.

Preparation of the Medium

High water quality (i.e low salt and/or low amount of organic or chemical impurities) is important for growing cultures and test of the water supply used for growing cultures should be determined.

To prepare media, use a large pyrex flask or beaker and add a portion (one half to the two third) of the final volume of water. Sugar may be added at this time or not until the end. Stir add by pipettes or a graduated cylinder proper amount of each ingredient from stock bottles agar is added next and then the solution is made up to the prescribed volume by adding water (Chu,1986). Adjust to the desired pH (usually about 5.7), with drop by drop additions of 1N HCL or 1N NaOH using a pH metre (or pH indicator strips) to measure.

The solution is heated to melt the Agar, this may be done in an autoclave at 151°C (218°F) for three to seven minutes or on a hot plate, stirring continuously to prevent the Agar from settling and burring. The hot solution is then dispensed into containers and sterilized in an autoclave for 20 minutes at 121°C. The greater the volume of media to be sterilized, the longer the sterilization time (Hudson, 2004). Substances that are unstable during autoclaving must be filter sterilized. The appropriate concentration of the material is added to the medium after autoclaving when the medium has cooled to 35 to 40°C before dispensing into the final containers. The medium should be used within two or four weeks and refrigerated if kept longer.

B- Aseptic Proceedures

-Disinfection:- Is the process of removing contaminants from the surface of the organ rather than from within the organ. Primary disinfectants include alcohol (ethyl, methyl, isopropyl etc, usually about 80%) and bleach (calcium or sodium hypochlorite, usually with 5.25 to 6.0 percent active ingredient) bleach is usually diluted to 10-20% before use. A few drops of detergent should be added to the bleach to improve surface coverage. A compromise must be developed through testing for the kind of explants to be disinfested.

A typical procedure for shoot-tip propagation is to cut the shoot in short pieces several centimeters long and wash in tap water with a detergent. For woody pieces a quick dip in alcohol may be helpful. Wrapping the shoots with small squares of sterile gauge will hold them intact during the treatment. In a hood, the plant parts are placed in to disinfecting solution, using a solution of 1-10% of the prepared material for 5-15minutes. The solution is then poured off; and the material rinsed two or three times with sterile water. Modifications of this basic procedure that may improve effectiveness include pre-washing, mechanical agitation, vacuuming and multiple treatments. (Hudson et al 2004).

Supplies needed for transfer operation are follows:-

Burner for sterilizing dissecting tools and containers.

Forceps to hold and manipulate tissue.

Dissecting needles with wood handles or a metal needle holder with replicable tips.

Dissecting scalpels, metal scalpel holders with replicable knife blades are most convenient.

Sterile Petri dishes can be used for holding explants and sectioning them.

A dissecting microscope and a top-loading balance these may be important for specific operations.

Micropropagation Procedures

There are basically four stages to the micropropagation process. These are:
- Stage I. establishment and stabilization
- Stage II. Shoot multiplication
- Stage III. Root formation
- Stage IV. Acclimatization

Stage I. Establishment and Stabilization

The function of this stage is to disinfect the explants, establish them in culture, and then stabilize the explants for multiple shoot development. This consist of

-Preparation for Establishing Cultures

Handling stock plants:- Two principal considerations are involved in handling stock plants. One is reducing the

potential for contamination by fungi, viruses and other pathogens. These pathogens are not automatically eliminated by *in vitro* technique unless this goal is built into the system. Preselected 'virus-free' or pathogen-indexed stock plants should be used. Otherwise special techniques, such as meristem-tip culture, heat treatment, and various culture-indexing tests, may be incorporated into procedure.

-Choice of explants:- The kinds of explants and where and how they are collected varies with the purpose of the culture, the species, and often the cultivar. Shoot tips should be collected from plants in relatively active growth. The size of the explants may vary from as small as 1 to 5 mm meristem tip for meristem culture to a piece of shoot several centimeters long. A single node bearing a lateral bud also can be used. For woody plants, a shoot tip from a dormant (but not resting) bud may be utilized but is often difficult to disinfect. Removal of the bud scales, plus cutting away the leaf scar, may give sterile tissue.

For woody plants, it may be helpful to collect branches in early spring before growth has begun and to bring the cut branches into a growth chamber or greenhouse to force growth. A florist's cut-flower preservative in the forcing water can improve growth. Collect explants from the expanding new growth. Explants collected in this way are easier to disinfect than those collected directly from new growth outdoors.

Pieces of leaves with veins present, bulb scales, flower scapes, and cotyledon also may be used for obtaining explants.

-Pre-treatment: shoots are disinfected by procedure describe earlier. Sometimes a preliminary treatment step is included. In a petri dish, the explants are placed on the surface of an agar medium of basic salts and sugar, but without growth regulators. The purpose is to detect contaminant of individual material and to control exudation of phenolics and other substances from the cut surface that inhibits development. Addition of activated charcoal, ascorbic acid, or citric acid to the medium can be used to overcome the problem. Using liquid media in the initial stage or frequent transfers on agar medium for several days may leach out the toxic materials. Likewise, using antioxidants, such as ascorbic acid and citric acid, in the preliminary washing may be useful.

Initial Shoot Development

Depending on the type of explant, shoots will initiate from(a) the stimulation of axillary shoots;(b) the initiation of adventitious shoots on excised shoots, leaves, bulb scales ,flower scales, cotyledons and the other organs; or (c)the initiation from callus on the cut surfaces. The medium selected varies with the species, cultivar, and kind of explants to be used. A basal medium (BM) include the ingredients listed earlier under in organic salts, plus sucrose and sometimes other supplements. Control of

development is most often achieved by manipulating the level of auxin and cytokinin. If axillary shoots are desired, a moderate level of cytokinin(BA, kinetin, or 2iP)is used(0.5 to 1mg/1).The auxin level is kept very low (0.01 to 0.1mg/1) or omitted altogether.

To develop adventitious shoots on explants(excised shoots, leaves ,bulb scales, cotyledons, flower scapes, etc.), a somewhat higher cytokinin level is needed, for callus formation, increased auxin level are needed but must complement an appropriate level of cytokinin. Usually four to six weeks are required to complete stage I and produce explants ready to be species transplanted to stage II.

Some woody plant species can take up to a year to complete stage I(McCrown, 1986).This is termed stabilization. A culture is stabilized when explants produce a consistent number of "normal"shoots after subculturing."Abnormal "shoots usually are arrested in development and fail to elongate.

Stage II-Shoot Multiplication

In the multiplication stage each explants has expanded into cluster of microshoots arising from the base of the explant. This structure is divided into separate microshoots, which are transplanted into a new culture medium. This is called subculturing. During the multiplication stage, cultures are subcultured every four to eight weeks.

The kind of medium used depends on the species, cultivar and type of culture. Usually, the basal medium is the same as in stage I, but often the cytokinin and mineral supplement level is increased. Adjustments may need to be determined by experimentation(Defossard, 1976).

Different species vary in the optimum size of the microshoot and method of cutting apart. There is generally a minimum mass of tissue that is necessary to produce uniform rapid multiplication in the next transfer. New culture are initiated in two ways from these development cultures. The elongated shoots are cut from the original culture and are subcultured as nodal explants. These may have the leaves removed and are typically two to four nodes in length .These shoots can be inserted into the medium in the vertical position or laid horizontally on the surface. Avoid pushing shoots too deeply in the agar and submerging the nodes. Elongated shoots laid horizontally on the other surface as a type of layering and stimulate lateral shoots to develop. After the elongating shoots have been removed and subcultured, the original explants can be further subdivided and recultured. Division may be made by vertically cutting the tissue mass into sections, keepimg some of the base with each piece. Sometimes one dominant shoots develops and inhibit eloghation of others. If this happens, the shoot may be cut off and the base recultured. For clump-forming plants(like hoster or day Lilly),all shoots form from a common base. These

crowns are separated in a similar way to standard division and each division is subcultured.

The number of microshoot produced ranges from 5 to 25 or more depending upon the species and condition of culture. Multiplication may be repeated several times to increase the supply of material to a predetermined level for subsequent rooting and transplanting. Sometimes microshoots deteriorate with time, lose leaves, fail to grow, develop tip-burn, go domant, or loss the potential to regenerate (Anderson, 1978).

During multiplication, off-type propagules sometime appear, depending on the kind of plant and method of regeneration. In some plants, such as Boston fern, restricting the multiplication phase to only three p;ropagations before reinitiating the cultures is recommended to avoid development off-type microshoots. Other procedure that can reduce the potential for variability include reducing growth regulator concentration and avoiding basal callus that may give rise to adventitious shoots in subculuring (Geneve, 1989).

Stage III-Root Formation

Shoots developed during the multiplication stage do not usually have roots. There are some exceptions(Like African Violet) that spontaneously roots on the multiplication medium. For most other species, single shoots, (microcuttings) must be moved to a medium or suitable environment to induce root. Therefore the purpose of stage three is to prepare the microplants for transplanting from the artificial heterotropic environment of the test-tube to an autrotropic free-living existence in the greenhouse and on to their ultimate location(Aitken,1991).This preparation may not only involve rooting,but it also may involve conditioning of the microplant to increase its potential for acclimatization and survival during transplanting, For example, the agar/ or sucrose concentration might be increased. Light intensity is sometimes increased during this period.

Microcuttings can be rooted either *in vitro* or *ex vitro*(Anderson,1978).For *in vitro* rooting, individual microcuttings are subcultured into new container in a sterile medium with reduced or omitted Cytokinin, and increase auxin concentration, and often reduced inorganic salt concentration. For some plants, rooting is best if the microcutting is kept in the auxin medium for only one or two days and then transferred to an auxin-free medium. Or the microcutting may simply be dipped into a rooting(auxin) solution and inserted directly into an auxin-free medium. Some plants root best if placed in the dark during the auxin treatment period.

Some plants respond to an "elongation" phase between stages II and III by placing the microshoot into an agar medium for two or four weeks without cytokinins(or at very low level) and, in some cases, adding (or increasing) the

gibberellic acid (Anderson, and Carstens, 1977).This reduces the influence of cytokinin. The microshoots are then rooted in a cytokinin-and GA-free medium.

Selection for uniformity and roguing of obviously abnormal, aberrant, or disease microshoots should be made prior or stage III(Anderson, 1980).Plant species having an inherent dormancy or rest period may need to be chilled to stimulate new growth and elongation(Anderson, 1980).

Microcuttings can also be rooted directly under *ex vitro* conditions. Microcuttings can be treated with auxin as a quick-dip.or 'pulsed' with auxin by *in vitro* exposure to a medium with relatively high levels of auxin for five to ten days. After auxin treatement, microcuttings are "stock" into plugs (Broome, 1986) containing a greenhouse rooting medium (e.g, two parts peat to one part vermiculite) and placed in a high humidity environment to form roots. This procedure is the same as that used during standard cutting propagation except care must be taken to prevent the fragile leaves on the microcuttings from dying out during the sticking operation. Frequent misting prevents this type of damage.

Stage IV-Acclimatization to Greenhouse Conditions

Once plantlets are well rooted, they must be acclimatized to the normal greenhouse culture vessel and the agar is washed away completely to remove a potential source of contamination. Plantlets are transplanted into a standard pasteurized rooting or soil mix in small pots or cells in a more or less conventional manner. Initially, microplants should be protected from desiccation in a shaded, high-humidity tent or under mist or fog. Several days may be required for new functional roots to form.

Plantlets should be gradually exposed to a lower relative humidity and a higher light intensity. Any dormancy or resting condition that develops may need to be overcome as part of the establishment process.

CONCLUSION

The powerful combination of conventional breeding and biotechnology breeding has the potential of greatly enhancing experimental breeding in to its peak, by increasing resistance to pest and diseases of the experimental Plants, which leads to rapid multiplication of the desired experimental Plants to meet the need of the outside world.

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