Actual State and Practical Use of the Plant Production System using Tissue Culture

Donald P. Holdgate

ACTUAL STATE AND PRACTICAL USE OF THE FACTORY-STYLE PLANT PRODUCTION SYSTEM USING TISSUE CULTURE

D.P.Holdgate and E.A.Zandvoort
Integrated Plant Technology Applications, 29 The Fields, Tacolneston,
Norfolk NR16 1DG, United Kingdom, fax +44 1953 778156.

1 INTRODUCTION

Since 1966 tissue culture has been used as a tool for the production of disease indexed stocks from selected plants and their rapid (clonal) mass propagation through the procedure now referred to as micropropagation. The major advantages have been the rapid introduction of new plant cultivars, created within conventional and mutation breeding programmes, as healthy stock for beneficial distribution and the expansion of the world wide horticultural industry.

The application of micropropagation has limitations because of the relatively high costs of operations. The nature and source of the real, local, high costs vary with country and regions, with labour, chemicals, energy and interests being the main items. In many cost analyses the facilities, equipment and their upkeep, and the true cost of capital are ignored. The price of micropropagated plants is generally higher than alternative plant supplies, and indeed may be beyond the financial resources of many growers and farmers. However the returns on the investment in these high quality plants can be vast, in terms of products and value. The fullest application should be made of tissue culture micropropagation to benefit all sectors and levels of crop development and production.

2 CONSIDERATION OF MICROPROPAGATION PROCEDURES.

2.1 What is Micropropagation

Micropropagation refers to multiplication of plants in *in vitro* via the proliferation of axillary buds, adventitious shoots or embryoids (embryo like bodies formed from somatic cells) arising from individual cells, callus, tissue, or organs, through the manipulation of nutrients, sugars, growth regulators, the growing environment and by mechanical subdivisions. The basic processes of micropropagation are illustrated in figure 1.

Cultures are initiated from meristems or other tissue from the selected parent plants. The cyclic procedure of micropropagation can be performed on solidified or in liquid media. Each initial developed culture should be tested as free from contaminating fungi and bacteria, and disease indexed as free from pathogens irrespective of the nature - viral, fungal or bacterial. The initiation of pathogen free cultures in general requires meristem tip culture, sometimes combined with heat treatment of the mother plants. Cultures from the majority of crops can be maintained as stock without subculture for a long time at low temperatures.

Axillary or adventitious shoot production is the procedure generally used in European tissue culture laboratories. The initiated cultures are multiplied up to a base stock from which shoots are transferred to the greenhouse, either as rooted plantlets or as microcuttings used for *in vivo* rooting. Alternatively whole cultures, rooted plantlets or unrooted shoots may be sold directly. Variations on the basic procedure can be found in crops such as potato and lily where the final product can be a storage organ, microtuber or bulblets, instead of a plantlet.

Another production procedure is through embryogenesis, in which case cell suspensions are multiplied in bioreactors. Through a change in medium or environmental conditions the cells are triggered to form embryoids, which can be dried and encapsulated to form an artificial seed. This prodecure has not yet been developed beyond the pilot commercial application stage for most crops. In the case of oil and date palm, procedures for multiplication through embryogenesis have been developed, but careful selection of suitable clones and culture form is necessary to avoid abnormal plant production.

The main areas of work and cost accumulations in the commercial micropropagation process are in the initiation and disease indexing phase, the cutting and transfer of cultures, the preparation and sterilisation of media, and in the acclimatisation of propagules. The relationship between these activities is explained through figure 2.

2.2 Commercial production operations

The majority of commercial operations function from purpose designed buildings allowing the incorporation of all necessary facilities, to perform all the basic activities including:

- initiation, disease indexing and general laboratories
- personnel changing areas
- washing-up and sterilising area
- media preparation kitchen
- inoculation room - for culture transfer
- growth rooms
- administration areas

The large labour force, and the high quality environmental conditions required demand thoughtful laboratory designs. Improper logistics or insufficient sterility can rapidly lead to culture contamination and total destruction of production. General hygiene of the laboratory is an important issue, requiring not only very strict routine cleaning procedures, but also the strictest discipline of all employees. Many laboratories will have routines for washing and changing of clothes prior to personnel entering the designated "clean areas" . Goods and plants entering the facility must be treated with similar care.

Media preparation is mainly mechanised. Sterilisation procedures are generally based on heating to high temperatures under pressure. The containers used for culturing are extremely

variable in design and materials, both between and within laboratories. Containers may be made from glass, autoclavable or non autoclavable plastic. The choice of containers, frequently made from the food packaging industry, is dependent on the sterilisation system to be employed.

The growth rooms of a modern micropropagation laboratory will have air conditioning systems to maintain specified temperatures during the light and dark phases. Unfortunately general growth room and air conditioning designs are inadequate resulting in poor ergonomics and extremely variable temperatures and light intensities which have a major adverse effect on plant quality.

A greenhouse is an extremely important, but often neglected, area of the facility. The handling procedure for the micropropagated plantlets of all plant groups is essentially common. Plantlets, or root-initiated shoots, or shoot microcuttings generally from a semi solid medium are transferred to a compost mix or manufactured plug, in a high humidity facility with adequate lighting to secure good root and shoot growth. Acclimatisation to the *in vivo* condition can be achieved through the progressive reduction in humidity over a period of 10 to 20 days but any plants lost in the greenhouse area are high cost losses. Aspects of the *in vivo* condition are discussed later in this paper.

3 MICROPROPAGATION IN EUROPE

3.1 Production levels.

A recent survey by the authors of commercial micropropagation activities in countries of the European Union, Norway, Switzerland and Poland was compared with those made in previous reports by Pierik (1991) and O'Riordain (1992), to generate an overall scenario in the development of commercial applications and an estimate for future trends in activity. It is unfortunately impossible to obtain exact data on the whole industry consisting of 200-plus small to large operations. Many companies, including breeders, wine, fruit and commodity producers, using tissue culture as an integrated part of their operation, or, as a specialised contract micropropagation service, do not publish figures concerning their production levels. Despite the lack of detail from such sources estimates have been based on data from available trade statistics and personal contacts, but it is accepted that some specific crops may have been missed, under or over assessed. Such errors would be limited to the more recent crop introductions and, be of relatively low volume levels and do not negate the trends identified. The order of magnitude of production by country is given in figure 3. Total production for the countries in Europe was at least 250 million plants in 1993.

3.2 Plant types.

The ornamental sector has been by far the most important for total micropropagation activity (figure 4). The pot plants (foliage and floral cultivars combined) make up the majority

of this sector, however since per unit price can be extremely low it does not follow that pot plants represents the greatest financial value. The plants for cut flower production are of substantial importance in The Netherlands and Poland. Fruit, which includes rootstocks and soft fruits, is the second largest product sector. Bulbous crops account for about 10 percent of the total volume, whilst smaller levels of production are found in the shrubs and garden plants, although these may represent very high levels of production in specific countries. The remaining production is represented by vegetables and some specialist items.

The use of plants in the various groups differs substantially. The pot plants, in general, are grown on for a short period (6 to 14 weeks) by specialist growers and then retailed directly via shops and garden centres, or through well established wholesale distribution systems. The cut flower group is sold internationally as young plants to growers for cultivation for cut flower production for a matter of months (*Gerbera*), or up to several years (*Anthurium, Cymbidium*). In this case the plant acclimatisation may be performed by the micropropagator, the original breeder as the main controlling sales outlet, or by specialist growers. Demands are more stable than for the pot plant group because cut flower growers tend to be growing a crop type for many years without change, and plan their planting regimes at least one year in advance. Many of the plants are protected by plant breeders rights, or by patent, and are only produced on agreed contractual terms. Prices tend to be higher than for pot plants and relate more to the added value gain over the alternative plants sourced from variable seed. For instance the micropropagated plantlets from selected cut flower varieties of *Limonium* sell for over US$ 2, as against 15 US dollar cents for seedlings due to the several-fold increase in returns based on flower stem quality, total yield, clear colour selections and market control.

Storage organs produced by micropropagation such as lily bulblets are used as mother stock for another one or two cycles of vegetative propagation before the bulblets are grown on to a size suitable for the specialist lily cut flower producer. Circumstances can arise where over-supply results in destruction of price. In the case of lilies massive over production mainly in low cost countries, led to a severe price collapse (60%) in 1993, and, worse for the growers, this was accompanied by a decline in health status and general quality. The urgent reorganisation and actions to reestablish standards has not been an easy task. This type of experience has stimulated more breeding operations to become vertically integrated in attempts to secure end product quality through total management control.

The fruit group of plants tend to be produced within integrated companies, although strawberries, for example, are produced for the use of general freshfruit producers and processing companies with own and contract growers, probably in several countries or regions, to spread harvesting times. Grape vine rootstocks are produced within the organisation of major vintners, registered top fruit (apple, pear, peach etc.) rootstocks may be micropropagated under licence for the open market. However more traditional producers micropropagate and grow to the whip stage prior to grafting for use in major plantation developments, or, to a lesser extent,

in direct sales. Bananas are being produced under contract by micropropagation companies in several countries for trading and plantation organisations of international fame.

Products within the other groups tend to be produced in low volume, either because of the nature of the product, for example vegetables such as *Brassica spp.* for seed production, or due to the relative infancy of the current performance trials, of for example *Asparagus*. Potato micropropagation is still limited in some European countries and is more related to the well established traditional propagation procedures involving a chain of farmer producers than the logically most efficient production route. Application to the forestry industry has been limited to trial scale operations with *Betula*, *Eucalyptus* and certain pines although the current trend in pulp prices appears to stimulate further tissue culture activity to initiate faster realisation of bred genetic gains. There has been significant interest in the micropropagation of biomass crops such as *Miscanthus* during the past 5 years.

3.3 Trends

Production in 1988 is compared with the estimates for 1993 in figure 5. Production levels in Italy, France, Belgium and Scandinavia appear the be rather stable, whilst Germany and Spain increased production. The United Kingdom lost some laboratories, resulting in a reduced production. Poland is becoming a significant producer, mainly due to operations set up under Dutch and Germany management. The situation for The Netherlands looks optimistic, but the real pessimistic picture is shown in figure 6. Until 1990 there was a steady increase in the number of tissue culture operations and in total production. There are no definitive data for 1991 and 1992 but there was a dramatic change with total production falling by at least 15% by end of 1993. The decline has continued and many Dutch companies have closed. The reasons for the decline are real reductions in demand for important lines (*Gerbera* and lily), and the very severe competition from low cost countries, India, Poland, Bulgaria, Hungary, Malaysia, and the USA. Some of these imports were produced by Dutch breeders and tissue culture operations themselves having arranged facilities in low cost countries in efforts to capture higher market share. The economic situation forced other growers to seek the lowest cost plants, inevitably from non-European facilities. In general product quality from low cost sources has not met the standards needed so that both the growers and micropropagation operations have lost out; but the latter permanently. Such low cost competition will continue since there are more would-be-suppliers every day from an increasing range of countries.

3.4 Future Developments.

The expanding competition from low cost countries has been disruptive for the Dutch

and indeed for the whole European industry. The position raises a question on the future prospects for the European tissue culture operations and opportunities elsewhere. It is inevitable that there will be a continuing pressure on micropropagators, in all regions, to reduce prices of all product types. This will result in company closures in high and low cost countries. The highest quality micropropagation producers and those within vertically integrated operations in Europe will survive, and through innovation even expand output, whilst some production operations set up by marketing entrepreneurs will revert to the original selling function for other producers. However breeders of major products requiring micropropagation services have tended to become vertically integrated to link protection of product propagation and quality with varietal protection gained by Plant Breeders Rights.

The establishment and control of micropropagation operations in lower cost countries will also be sought by those in high cost and high market demand centres. Production capacity will be used to satisfy own small plant supplies, and, where appropriate, the needs of other growers. These investment exercises will be to secure management control of an essential part of their business, and to fit product specification with the technical advances and any phytosanitary or other regulatory requirements in the ultimate end product production centres, for example Germany and The Netherlands for cut flowers.

The use of tissue culture is common in major European seed companies. Applications are linked to securing rapid market entry of new cultivars and the generation of novel cultivars through interspecific hybridisations and genetic transformations to enhance both agronomic and processing traits. Regeneration and rapid propagation of plants from genetically transformed tissue is important for completion of trait performance and agronomic trials. For vegetatively propagated crops reliable and economic systems for producing and delivering genetically transformed planting material to the farmers must be obtained, and tissue culture based systems may be of major value for many crops. Several major breeding and seed companies are active in long term research and development studies in the control of embryogenesis and the production of artificial seeds.

Although the production of ornamental crops has dominated the European and USA micropropagation markets, the nature of requirements in developing countries is different. Food, plantation and forestry crops are of greater importance so that the percentage of total production covered by these crops is significant. The total production of food crop plants through micropropagation in the Asean countries in 1992 was estimated at 7.5 million plants, of which banana accounted for 6.9 million. The total production of ornamental plants was 8.3 million, and that for the industrial/forestry plants accounted for 1.8 million plants (Eldiningrad, pers. comm.). The trend of increased production through micropropagation of food and industrial crops is continuing and at an increasing rate with the more global acquisition of the technologies. In tropical and sub tropical areas disease free multiplication of the important

vegetatively propagated food crops is a major difficulty and requirement. Disease indexed culturing linked with micropropagaton and relevant nursery techniques to secure good quality nuclear stocks could make major contributions to agricultural productivity. A further requirement for the application of micropropagation in these countries is the need for fast introduction of improved germplasm (disease resistance, improved yield and nutritional value) of food crops, which can only be achieved by incoporating micropropagation in the traditional clonal multiplication procedures. Significant challenges and opportunities exist.

After some 30 years of active research it is reasonable to predict a broader application of micropropagation within the world-wide forestry sector in the near future. The approach which will be employed to maximise returns from the genetic gains being identified in breeding programmes will depend on a number of factors, and is likely to vary with region and tree types. Micropropagation procedures for selected clonal stock plants will be appropriate for some situations, but others may require the use and propagation from limited numbers of seed, derived from careful hand pollinations, to increase the number of genetically superior offspring. In both these cases the micropropagation may be limited to the creation of an elite nuclear mother stock to be grown-on as a source of traditional cuttings for *in vivo* rooting, to secure the genetic gains from the breeding programmes.

4. AUTOMATION AND MECHANISATION IN MICROPROPAGATION

From the start of commercialisation of plant tissue culture for propagation attempts to develope systems and apparatus to mechanise handling operations have been encouraged even if not generously financed. Each part of the sequence can be considered separately.

4.1. Initiation

The actual initiation of cultures by dissecting meristems from apical and axillary buds of the parent plant is a highly skilled activity and has not been the subject of automation or mechanisation. The diagnostic procedures adopted however have been highly mechanised. The use of special plant tissue presses to collect sap from small samples on an almost continuous basis without cross contamination of samples has become routine in Europe. Not only does this reduce tedious manual procedures and increase the number of samples to be handled in a given time but it also facilitates more economic and accurate diagnostic analyses. Equipment for mechanisation and automation of aspects of diagnostic procedures such as ELISA (enzyme linked immunosorbent assay), PCR (polymerase chain reaction) and RAPD (randomly amplified polymorphic DNA) are readily available, and can fit the level of sophistication justified by operational size. Diagnostic servicing laboratories operate with a substantial level of automation.

4.2. Media preparation.

Media preparation was one of the first areas in which mechanisation and automation was developed. The general steps in media preparation are:

- mixing of chemicals and water, pH adjustment
- sterilising of media
- dispensing of media in appropriate containers
- labelling and storage

Dissolving and mixing of chemicals to produce concentrated stock solutions for medium preparation is done in general with the aid of an impeller type mixer. Heat sterilisation can be performed in:

1. the culture vessels, which must then be made of glass, polypropylene or polycarbonate to withstand the temperatures,

or 2. in bulk, followed by pre determined volume dispensing into pre sterilised (heat, irradiation, gas sterilisation) containers usually of low cost plastic,

or 3. semi-continuous procedures allowing collection of required media volumes in sterilised containers.

Growth regulators which may break down at high temperatures may be added through filter sterilisation after the medium starts to cool. Dispensing of media into containers can be partly manual or totally mechanised, with automated filling and closing of containers. Media preparators, designed to mix, heat-sterilise and dispense, have been made for a number of years. These reduce the amount of labour required in medium preparation, sterilisation and dispensing to a maximum level of four people in a major commercial operations.

4.3. Cutting and transfer.

The cutting and transfer of cultures under sterile conditions presents the greatest challenge for mechanisation and automation. Attempts at automation have been the subject of studies in UK, USA, Israel, Australia, Japan and The Netherlands.

In general, manual cutting and transfer of cultures includes the following handlings:

a transfer from growth room and examination of the container for contaminated and / or abnormal growth

b opening of containers

c removal of the individual cultures

d selection of cultures
e presentation of cultures for cutting
f removal of dead plant material, trimming of cultures
g cutting of cultures into desired explants and grading (size, form, origin)
h discarding of waste and old culture containers
i planting of explants in new containers of appropriate medium
j closing of containers
k sterilisation of instruments before re-use
l labelling of containers and transfer to growth room
m cleaning of working surfaces

Detailed activity studies have shown that the aseptic transfer handlings (c - i) amount to less than 40% of the total labour requirement, the other 60% is for " non-productive" activities a, b and j to m. Nearly all the culture steps include decision making and actions under sterile working conditions: where to hold the culture, how and where to cut the culture, and what to do with the cut explant. These requirements present major problems to the automation engineers.

Some developed systems have tried to utilise biological characteristics of the plant to try to simplify the culture subdivision / transfer procedure. A completely mechanised separating and transfer system of bulb scales for propagation of lilies is described by Takayama et al. (1991). Lily bulblets produced *in vitro* are mechanically placed, one by one, onto two rollers that remove the roots. The scales are separated then by pressure at the, more or less, natural breaking point at their junction with the basal plate. Separated scales are transferred to a fresh medium by a robotic hand controlled by a vision system.

Mechanised and automated cutting systems based on nodal cuttings have been claimed by a number of sources. Shonstein and Johnson (1986) describe a system to subdivide and transplant shoot material. Individual shoots are removed from containers, placed on a sterile conveyor belt, and a computer image is taken to establish the location of where the cut has to be made for subdivision of the stem into nodal sections. After cutting with a double bladed knife, the nodal sections are placed on fresh medium for the next cycle of growth. This system is under further development by an Australian company. A robotised scisser-based nodal cutting system, developed by the Japanese Toshiba Company (Fujita 1989), gives a further example of image analysis linked with automated cutting but the tools used are very slow and would have very limited application at the commercial level.

The authors have participated in the development of an integrated automated system using a computer aided laser beam for cutting, and simple robotised pick and place units for planting. The whole could be linked to control a total production mangement system (Holdgate

and Zandvoort 1992). The basic objective of this system was to develope an automated procedure capable of producing in the region of 10 million microcuttings of constant high quality per annum. To achieve this the cutting and handling tools must reach minimal criteria in respect to:

- speed
- accuracy and consistency
- reliability and longevity of use
- access to areas to be cut
- decontamination of the cutting tool
- maintenance of overall sterility
- limited damage of cut surface, subsequent growth of the explant, and eventually plant uniformity and quality

Some cutting options (blades of metal or ceramics, water, mechanical shearing) were rejected because of the anticipated problems with sterilisation of the tools, or the lack of precision considered necessary for the majority of crops of interest. The option identified to offer the most positive potentials in terms of accuracy and speed, without sterility problems, was the use of a computer controlled laser beam for cutting. Initial trials to determine the unknown effects of laser cutting on the growth of explants demonstrated normal to enhanced shoot growth and rooting of explants, providing the power of the laser was high enough to secure a rapid, smooth cut.

The experimental laser cutting tool, based on a variable power energy source to allow adjustment of the power of the cutting beam to the type of plant and tissue to be cut, had to be controlled by the cutting algorithms developed. An image analysis based on a two dimensional picture of the plant to be cut was not accurate enough to provide the precision and efficiency required. Bud damage or failure to cut some nodes was high. The subsequently developed three dimensional image analysis systems gave total satisfaction. The basic data for the algorithms were provided by the biologists participating in the team that developed the system.

The involvement of the biologists with specialised detailed knowledge of propagation and commercial production of plants secured the essential link between the requirements of the commercial micropropagation operation and the various automation engineers developing the cutting system. The multidisciplinary character of the development team facilitated the solving of major problems that had to be overcome:

presentation of plants to be cut to recognition and cutting systems in the correct position without damaging the plants

- recognition of the points to be cut and development of the algorithm for cutting
- serial recording and collection of the cut explants, transport according to type
- collection and disposal of cut-waste
- development of algorithms for detecion of position of cut explants, required to operate the pick and place unit used for planting explants
- precision picking and placing of cut explants in new containers
- sterilisation of mechanical parts in contact with plant tissue

The major operational targets for the mechanical engineers were the speed of operation, set by the basic output requirements, and the biological requirements set by the conditions required for the plant to retain optimum status to achieve growth performance. The biological requirements included the need to maintain a specified operational environmental temperature and humidity, a total time limitation from shoot entry to completion of operations, limited time available between cutting and planting of explants to prevent drying of explants, prevention of damage to plant tissue due to mechanical pressures during transportation, accurate placing of explants, and the already mentioned accurate cutting and overall aseptic specifications.

The prototype of the laser cutting system performed the following sequences:
Grown shoots are carried to a camera recognition system by simple grippers mounted on a circular conveyor belt. A three dimensional picture is taken by rotating the free hanging plant twice by 90 degrees, and the data are processed according to the provided cutting algorithm. The plants are carried further to the cutting position, and cut by a laser beam, according to the calculated cutting lines, again with access from two different angles. The shoot tip and nodes are collected singly in cups mounted on a conveyor belt and transported to a pick and place unit. Cut waste is removed from the system. At the pick and place unit another image is taken of the cut explant to determine the position and orientation to prepare computer guidance for the retrieval of the explant and precise placing in fresh medium. The filled containers are automatically closed and labelled prior to transfer to the growth room.

This automation system of the micropropagation procedure allows for the collection of highly accurate information for use in management control of plant production within the industrialised concept behind the project. Each and every step in the process is recorded, stored and integrated to provide a continuous mechanised production system with performance schedule checks. The collection of data linked to parent plant explant origin and subsequent growth performance opens up the opportunities for total production control and progressive planning of necessary future actions. The ongoing comparative analyses of selected growth parameters within routine production and experimental conditions provides valuable information applicable to future culture and post culture activities to maximise quality and yield performance. Due to strategic changes by the parent company the development of the laser cutting system did not

proceed beyond the prototype stage and has not entered commercial operation.

A similar stage has been reached for a cutting system capable of sub-dividing clusters of plants in a best-option analysis with the aid of an image analyser in the United Kingdom. Cutting in this case is performed by a multiple cutting edge tool which is controlled through the analysis of the culture image. Within the cutting process the entire clump is retained within the cutting tool and transferred to the new container of media where individual cut pieces are sequentially planted.

In Israel scientists took a different approach to mechanisation of many plant types. They have concentrated on securing high multiplication rates in liquid culture followed by random cutting and plating pieces onto a semisolid medium. Since the rate of multiplication is high, and the cost of individual shoots low, loss at the cutting point does not cause significant valuation loss. In cases of high valuation of the *in vitro* stage biological imputs are employed to secure the ideal uniform growth of shoots which are then chopped into approximately uniform segments on a conveying line while colour computer vision is used to identify and locate segments with viable buds or shoots for automatic selection for subculturing.

Automation achievements reviewed (Zandvoort and Holdgate, 1991, Holdgate and Zandvoort, 1992), have indicated some principles of success, but no one system has been commercialised. The reasons may be found in:

- withdrawal of financial support for research and development
- over design, leading to unacceptable costs for facility and equipment
- slow speed of operation inherent in some lower cost approaches
- insufficient business volume to warrent commercial uptake within a national system
- inadequate links between mechanical engineering and commercial micropropagation to achieve appropriate and relevant advancement
- loss of relevance to the developing company
- failure of commercial micropropagation companies to agree terms and make the capital investments to complete operational developments
- total capital cost of retooling and reorganisation being too great

5 FURTHER ADVANCES IN MICROPROPAGATION

Major limitation of current micropropagation procedures whether in so-called high or low cost countries is the unit product cost. Whilst an attractive European market may be experienced for ornamentals by a few Indian producers, and sought by many, real consideration should be given to the relevance of micropropagation to local crops and farmers. Where application must address National production levels a position equitable with the situation in the

European operations will be found, although unit labour may not be the highest cost or limiting factor. All inputs, labour, utilities, chemicals, transport, selling, communications, management and all overheads must be incorporated into costs calculations. The nature, duration and sustainability of the production programmes must also be assessed by the contractor.

A percieved expansion of micropropagation to incorporate requirements in food and fibre crops besides horticultural crops will affect the type of production criteria needed. The horticultural sector is a sector depending on fashions like colour and shape of flowers. New varieties therefore have only a short life expectancy, and the market requires a rather wide range of varieties. The required planting material is consequently in relatively small numbers per variety. Food and fibre crops are more universal in their qualities, and good varieties or clones are likely to cover large areas for a substantial number of years. The required production of planting material will be larger volumes of the same variety. Current micropropagation procedures with low multiplication factors are unlikely to be efficient in meeting the high volumes in the required delivery windows for farmers. The automation approaches developed in the industrialised countries have led to substantial reductions in labour requirements and increased efficiency levels in media preparation and sterilisation, but the cutting and transfer of plants is still a manual task. Future automation activity must be targetted to:

decrease labour input
secure a general reduction in costs
create products which can be efficiently handled at the subsequent level
increase output per cubic meter of controlled environment space
improve ultimate end product quality

All these actually relate to the consequences of periodic subculturing onto semi solid media within defined containers with limited nutrient capacity. The conventional tissue culture procedures in which limited multiplication factors are achieved (two to five every four weeks), require high efficiency of cutting and aseptic transfer since losses of material due to wrong cutting or contamination will be very serious in terms of yield, the costs, and completion dates.

The engineers working on micropropagation during the last decade had the disadvantage to be in competition with very low labour cost businesses, market dumping procedures, and local over-production which combined to present unattractive investment scenarios. Present and future developers of automation programmes should take advantage of recent researches to integrate the latest advances of mechanical, automation and biological sciences and previous achievements to develope systems compatible with the growing market requirements and sitings.

The original procedures employed in the earliest commercial applications of micropropagation were in the form of liquid cultures. For the propagation of orchids and

pineapples rotating batch cultures were employed, whilst meristem cultures to secure disease indexed plants utilised filter bridges standing in the liquid medium; a system still empoyed by some facilities today. More recent applications of liquid culture procedures have been demonstrated for the propagation of *Lilium* (Takahashi et al. 1992), potato (Rosellet al. 1987), *Gladiolus, Nephrolepis* and *Phylodendron* (Ziv 1991), pines (Pâques et al. 1992) and *Alstroemeria* (unpublished research of the authors). Although the products of these liquid culture systems are not yet to the standards required for commercial production and further development will be necessary, multiplication rates have been generally good to exceptional.

The conventional micropropagation procedures often provide for rooting *in vitro* by a last transfer to a medium with auxins. Rooting, however, is the most expensive aspect of *in vitro* propagation for complete plantlet production. Preparation of cultures for rooting is time consuming and the required growth room space is expensive. Root initiation at the final stage of multiplication by addition of auxin has been advocated, but this can result in a wide variation in response and plantlet size. The removal of the rooting phase from the laboratory can reduce *in vitro* activity by more than 50 %, and potential cost savings can be high. Furtermore *in vivo* rooting provides for undisturbed shoot and root growth within nursery mechanisation operations.

The available data strongly suggest that:

- modification of the procedures in the multiplication stage to effect a superior proliferation rate is feasible

- transfer of many activities to high quality *in vivo* conditions are desirable, effective and cost beneficial.

A major shift to liquid culture for the multiplication phase, followed by high quality *in vivo* culture for rooting, acclimatisation, and any further proliferations required, has favorable features. A number of system options for liquid culture are available:

A. Total immersion with supplied gaseous exchange capacity and culture agitation.
These systems provide for a number of options concerning the aeration and agitation of the cultures. Commonly used systems are the airlift fermentor with aeration and agitation by air bubbles released at the bottom end of the container. Oxygen and CO_2 levels of the medium can be controlled by the composition of the air used. Impeller agitated systems have been widely used in microbial culture, but appear to be less successful for plant and organ cultures because of shear damage. Rotating drums have been applied for lilies (Takahashi 1992) in large bioreactors (up to 2000 l), and bubble free aeration using silicon tubing, in combination with vibration stirring has been successfully applied by Preil (1991).

B. An intermittent contact of the culture with the medium through an ebb and flood

Tisserat and Vandercook described in 1985 a microcomputer controlled system in which cultures positioned in compartments in a polystyrene chamber were intermittently immersed in liquid media by flooding. Recently the positive effects of flooding and aeration sequencing on the growth of cultures were confirmed by Teisson and Alvard (1995) for several tropical crops.

C. Floating rafts

Microporous rafts, allowing uptake of underlying liquid medium with the advantage of possible transfer of the complete raft to fresh medium, have been subject to several studies. The advantage of full aeration of the plants is combined with the easy uptake of liquid medium. The applications of this system, for which purpose-designed containers with rafts are available, fit into the conventional micropropagation procedures based on small containers, but the full advantages may be in large scale automated exchange of medium and transfer of cultures.

The *in vitro* options may be directed to traditional procedures like axillary and adventitious bud proliferation, but are also important for embryogenesis, and other forms of multiplication like proliferation of cell clusters prior to organogenesis. Autotrophic growth during the *in vitro* stage, omitting sugar in the medium and therefore reducing the contamination pressure has been demonstrated to have potential for a number of crops (Kozai 1991). Within certain conditions the procedure might prove of great advantage for some crops. However the abscence of sugar in the nutrient medium does not obviate the need for care at the subculture transfer since the cut surfacesof the plants are major centres for infection.

The advantages of *in vivo* rooting have not yet been fully exploited. The combined rooting and hardening requires more accurate control of the greenhouse environment, and experimentation may be required to optimise conditions and procedures for specific crops, but generalities are known. Composts should be open and well aerated, air movement should be good, and a high relative humidity should be maintained preferably through a fogging system. As an alternative to an organic compost mix mineral wool plugs of various manufacture are used extensively in European nurseries. Where an auxin treatment is required to secure root initiation this may be done in the transfer room in association with shoot preparation, or by dipping in an auxin solution or powder prior to transplanting. The rooting process can be generally linked with that of acclimatisation to normal growing conditions so that first transplantings are possible in 10 to 20 days.

The vagaries of climate may be eliminated by employing a specially developed growth chamber within which all aspects of growth can be controlled. The system can be ajusted to fit mechanised nursery handling systems and acclimatisation linked to the ambient nursery or outside conditions to be used on transfer.

5 TO A NEW CHALLENGE

This paper has reviewed the historical position of European tissue culture, the starting centre for commercial activities. The entry and maintenance of the major activities in the horticultural, ornamental and fruit, crops was deliberate for commercial survival. Attention has been drawn to the position and role of some so-called low cost countries on the changes and attitudes in the European activities. Although it is natural that some commercial operations have been established in these areas, as in India, to offer services to the relatively high value European markets, local needs should be recognised. There is a subtle but definite variation in the efforts and interests of tissue culture applications in developing regions of the ASEAN and South American countries. These needs incorporate the requirements for improvement in planting material of local food and commodity crops. There is a clear need for the application. The potential requirements in volume and product quality are demanding. The questions of how to achieve have been placed, particularly as automation has been of only limited success.

The conventional procedures, developed and used in commercial operations in Europe the USA and in some developing countries have demonstrated the limitations and inefficiencies of the unit capacity culture system. To satisfy the needs of a sustained industrial approach for future demands a new order of magnitude in productivity is required. It is our conclusion and submission that, to establish a tissue culture programme or production facility to achieve this goal, irrespective of siting, basic studies should be made on:

- the crops of interest, to provide the definition of objective requirements to be secured from the application of the tissue culture systems

- tissue culture developments to identify the most promising techniques for crops of interest

- the *in vivo* requirements of the crops and selection of procedural options for exploitation, develpoment and application

- mechanisation and automation approaches with selection of appropriate parts

The conclusions should provide a solid base for strategic and developmental programmes required to secure the agricultural needs of the twenty first century.

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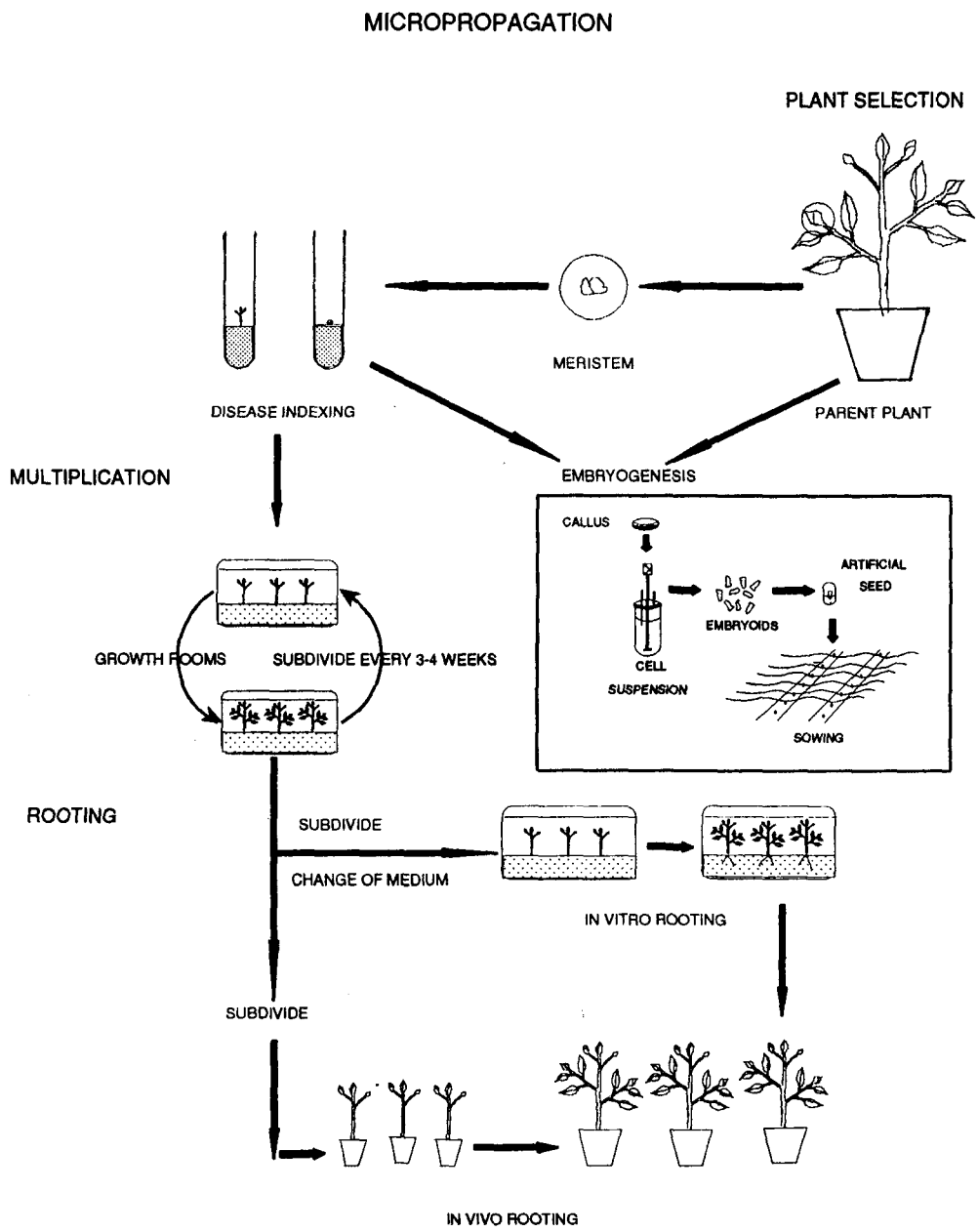

Figure 1 **Basic process of micropropagation**

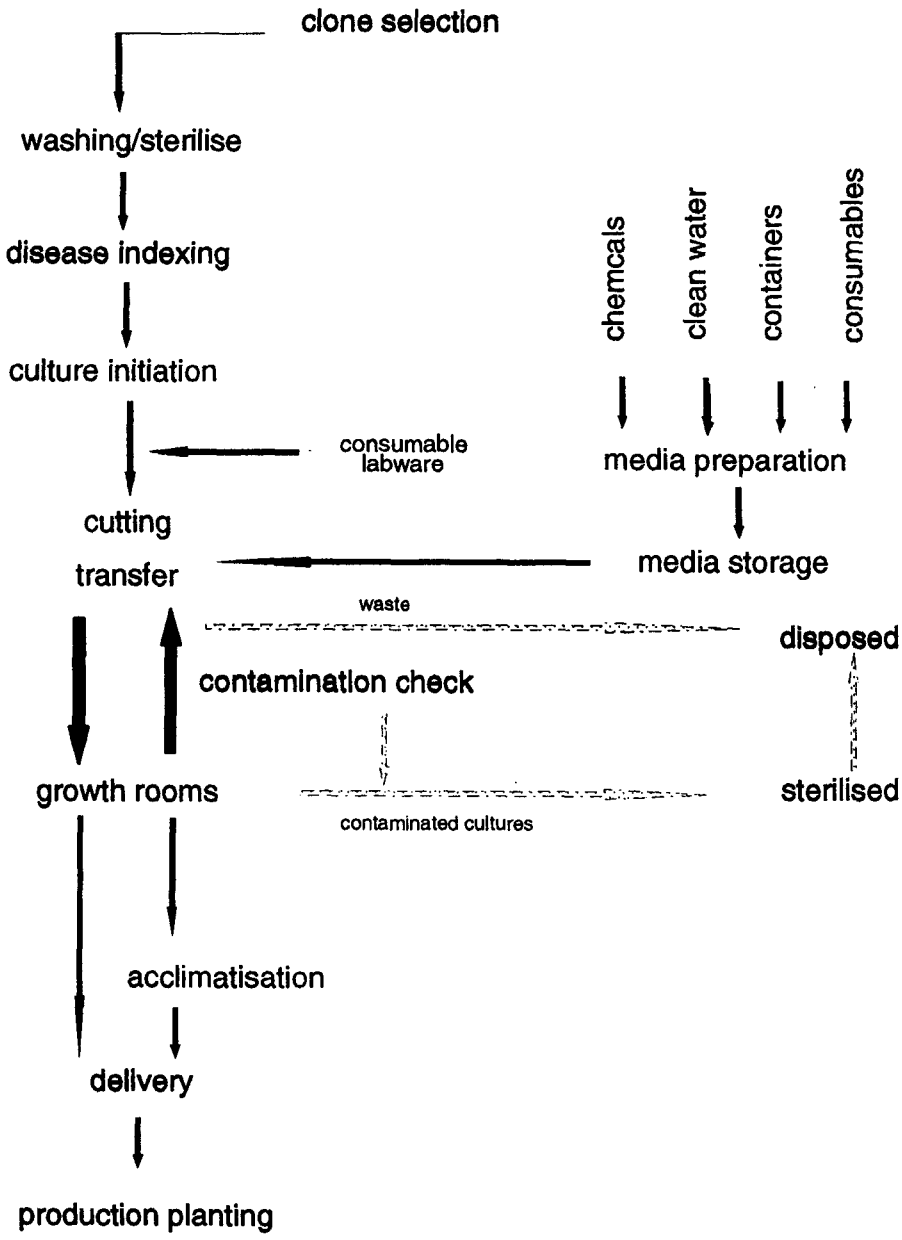

Figure 2 Basic inputs for micropropagation

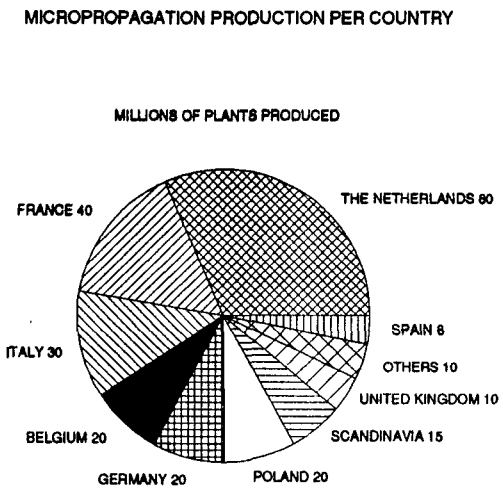

Figure 3 Micropropagation in Europe production by country in 1993

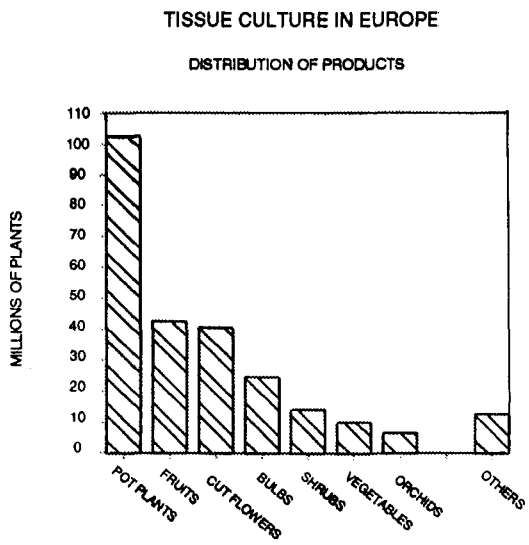

Figure 4 Micropropagation in Europe distribution of products in 1993

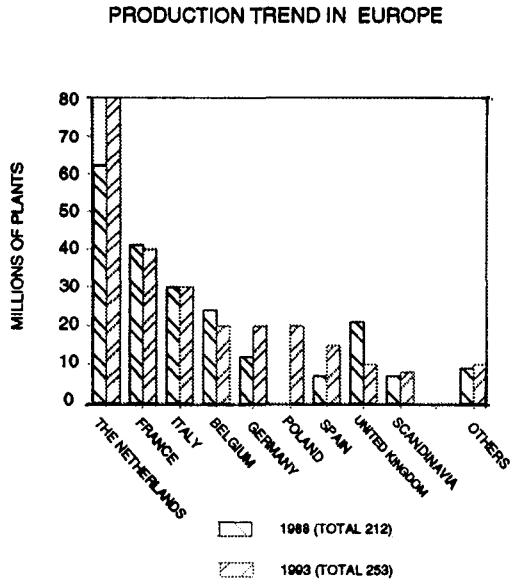

Figure 5 Micropropagation in Europe production trends

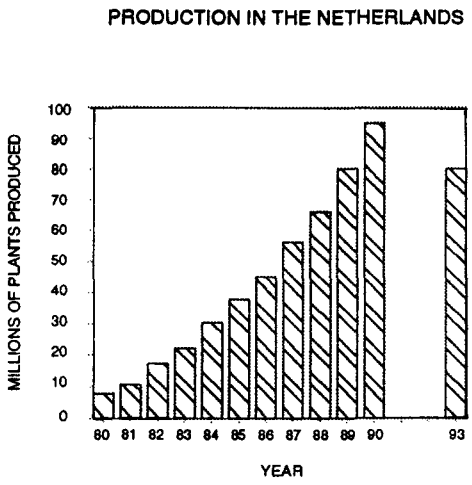

Figure 6 Micropropagation in The Netherlands, production trends