Automation in plant tissue culture: problems and prospects

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Commercial exploitation of tissue culture technology is limited to only a few species because of the high cost of production. Labour and media constitute more than half the cost of a tissue culture operation. This can be reduced substantially by devising systems that use liquid media. Bioreactor technology can be used to produce somatic embryos en masse. Automated systems are also available that can be used for micropropagation through axillary buds.

The plant tissue culture or micropropagation is a method of propagating disease-free clonal plants. Plant tissue culture is favoured over propagation by conventional means such as by cuttings or seeds as it offers various advantages[1]. Usually micropropagation can be achieved in three different ways: (i) by direct organogenesis via shoot tip or axillary buds, (ii) by indirect organogenesis through callus and, (iii) by somatic embryogenesis directly or indirectly.

The number of species that can positively be tissue cultured has increased steadily over the years[2] and enables most of higher plants to be cloned rapidly. In addition to becoming an important research tool, micropropagation now has important practical applications in plant breeding, e.g. production of homozygous lines and novel plants with desired traits via somaclonal variation.

Plant tissue culture techniques, however, were developed initially to understand the nutritional and morphological aspects of plant development. With improvement in media and other manipulative techniques, it has amplified its scope and now plants are micropropagated at commercial level. It has already become a multi-billion-dollar industry producing vast numbers of plants.

In recent years a large number of micropropagation companies world over have emerged. However, not all the companies are generating good profits. As a result micropropagation industry has not grown appreciably.

The high cost of trained personnel, particularly in developed countries, is the limiting factor for the development of this industry. Commercial laboratories so far, have been using the procedures developed at research level in which skilled operators manipulate explants and shoot clusters in sterile air hoods. These methods limit the total numbers of propagules a person can handle. This restricts its application to plants in which a high unit cost of production can be economically justified. In addition, current method of tissue culture is difficult to automate. If automation is to become a reality the current tissue culture techniques need to be reviewed. Many significant biological and culture problems must be solved before the technique can be amenable to automation.

A number of nutrient media have been devised[3]. A medium consists of inorganic salts, an energy source, certain amino acids, growth regulators, vitamins and other supplementary compounds that may be necessary for a particular species. The most widely used medium, however, is the one devised by Murashige and Skoog (referred as MS)[4] developed for tobacco callus culture. It also supports the growth of many different species of plants. None of the media, however, has been fully optimized. Medium constitutes an important part of the total micropropagation cost (about 20%) and therefore requires special attention.

Agar is used in the medium as a gelling agent and to give support to explants. However, agar is expensive (Sigma agar costs $ 56 a kg) and binds nutrients which cannot be reused. Sucrose, on the other hand, is provided as a source of energy but it favours contamination which is the biggest challenge in the tissue-culture industry. Despite all aseptic techniques, cultures invariably show contamination. Losses due to contamination are tremendous[5]. Antibiotics and fungicides have been used without much success. Moreover, prolonged exposure to antibiotics/fungicides is not recommended as it is phytotoxic and also leads to the development of resistant microorganisms. Therefore, methodology is required to combat this problem. Sucrose makes in vitro plants photoheterotrophic, which creates problems, particularly when such plants are transferred to soil, as, at that time, the only source of C must come from photosynthesis which is often defective in tissue-cultured plants.

To prevent losses due to contamination, usually explants are grown individually in sealed containers.

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This creates a very artificial condition in which humidity and gases build up which affect the morphology of the plants. *In vitro* grown plants usually lack epicuticular wax and have highly reduced cuticle. More importantly, stomata of such plants are often nonfunctional or poorly developed[6]. This causes excessive water loss due to cuticular and stomatal transpiration. With the result, a great number of micropropagated plants wither and die when transferred to soil directly. In some cases it results in 100% mortality[7].

In order to prevent such losses, plantlets have to go through a period of acclimatization in the glasshouse. Use of protective environment such as humidity tents, intermittent misting and shade have been suggested[8]. These procedures add to the production cost yet stomata do not appear to resume proper functioning[6]. Acclimatization and *ex vitro* rooting can account for greater than 40% of the total production cost[9].

It is, therefore, important to devise alternative methods of tissue culture. The production costs can be minimized by[10]: (i) increased multiplication rates, (ii) improved quality, and (iii) automation.

The following techniques have emerged as alternative ways of tissue culture.

Bioreactor technology

Bioreactor technology is quite sophisticated for microbial, mammalian cell culture and plant cell cultures where the final product is a secondary culture and plant cell cultures where the final product is a secondary metabolite. Bioreactors offer myriad advantages, e.g. increased working volume, possibility of controlling physical and chemical environment (pH, pO_2, etc.). Despite all these advantages, bioreactors have rarely been used for plant micropropagation except for a secondary metabolite from cell or microbial cultures. Since, the final product of a micropropagation cycle are plants their quality and performance are likely to be affected by bioreactor design and conditions. Nonetheless, bioreactors with modifications would help overcome the problems of manual labour and would also provide optimum growth conditions by controlling chemical and physical environments. Bioreactors have been developed for the production of somatic embryos[11–14]. A large numbers of somatic embryos can be produced in a limited volume and they do not require a separate rooting stage.

Table 1. Progress of bioreactor technology for somatic embryogenesis

Bioreactor	Species	Reference
Two-stage spin filter	Carrot	11
Propeller-stirred reactor	Alfalfa	14
Bubble-column reactor	Begonia rex, saintpaulia, syngonium, philodendron, *potato, lily*, tomato,	19
	fragaria	20
Spin-filter reactor	Poinsettia	13

A universal bioreactor does not exist and therefore, bioreactors should be designed according to the requirements of growth and multiplication of the species in question. The following are some points that should be considered while designing a bioreactor[15]: (i) Biological needs concerning temperature, pH, pressure, concentration, sterility, avoidance of cell damage and foam formation. (ii) Engineering requirements: defined mass distribution, intensive mass and heat transfer, high productivity, reliable measuring and control techniques, correct modelling. (iii) Economic aspects, including economic large size feasibility, low investment and operation cost, high safety and good environment compatibility.

The reactors can be classified according to the type of agitation systems: (i) Reactors with mechanical stirrer, (ii) reactors driven by air flow (bubble column/air lift bioreactors).

Reactors with mechanical stirrer

Typical stirrers are propeller and pitched blade turbine stirres that cause axial fluid motion. Flat blade turbines provide radial fluid flow whereas paddle agitators cause tangial and radial flow[16]. In order to optimize the conditions, the number of blades, their positions and ratio of diameter need to be studied. However, mechanically driven bioreactors pose problems with plant cells. The plant cells are sensitive to shear damage[17] and agitation may result in mass and heat transfer, particularly when aqueous solutions are used.

Reactors driven by air flow

In air lift reactors, air is circulated in the medium usually from the bottom of the reactor. Low circulation causes a homogeneous laminar flow whereas high circulation results in turbulence. These reactors are easy to construct and are preferred because of shear damage and less mass and heat transfer. However, bubble reactor leads to the formation of foam which adversely affects growth and formation of callus. In addition, it strips off certain useful volatiles, e.g. CO_2. The formation of foam can be prevented by the addition of anti-foaming agents in the medium, e.g. silicone oil or 0.1–0.3% polypropylene glycol. It is not clear from the literature whether these substances have any effect on the growth of the plants.

There have also been advances to aerate the medium without causing foaming. Techniques have been developed to disperse air bubbles by supplying oxygen bubble-free using tubing inserted into the bioreactors[13] or by using porous hydrophobic hollow fibre membrane made from polypropylene which move slowly in the suspension culture[18].

For the production of somatic embryos, bubble-column or other bubble-aerated reactors[19,20] or propeller-stirred[14] or spin-filter reactor[12] have been used predominantly (Table 1). Spin-filter reactor is favoured over other reactors because it allows the passage of liquid media without the cell loss. The spin-filter reactor comprises a hollow nylon core surrounded by a filter cartridge. The filter rotates by a magnetic stirrer and because of boundary effects it allows spent medium to pass through without becoming clogged by the cells. Thus, the medium can be replenished/replaced without cell loss.

There are, however, certain inexpedient features associated with somatic embryogenesis which should be appreciated during its production in bioreactors:

(i) Technique of somatic embryogenesis is limited to a few species only. Majority being propagated through axillary buds. Therefore, the bioreactor technique can be used for only those species where the technique for somatic embryogenesis is available.
(ii) Somatic embryogenesis involves callus phase, therefore, can the clonal uniformity of plants regenerated be questionable?
(iii) Somatic embryos need to be encapsulated in sodium or calcium alginate coats to produce synthetic seeds which are amenable to mechanical transplanting etc. This results in increased cost. However, somatic embryos can be sold after germination.

Automated systems for axillary buds

Propagation using nodal explants for axillary shoot multiplication is the most commonly used technique in micropropagation[21]. Here, multiplication and growth of plants proceed normally and no variation results. Plants produced tend to be more compact and full in habit. Moreover, the propagation of plants using nodal explants is the simplest and the easiest technique which is amenable to automation.

Systems already exist which permit automation of media preparation, handling and labelling of growing boxes, e.g. propamatic micropropagation system[22]. This system is essentially an automated laminar flow station that dispenses, mixes and injects aseptic liquid state agar media. In micropropagation, the use of robot is considered to do the task of subculturing etc. and to reduce labour[10].

However, special robotic mechanism is required to dissect and handle cultures which also requires sensory input and interpretation so that the robot can undertake various tasks. Moreover, dissection of plants, and their transfer requires considerable intelligence and dexterity which needs advanced robotic techniques. Robotic systems have been described which can detect, pick up and transfer plantlets[23–26].

It is unlikely, however, that robots can perform all the tasks of tissue culture laboratory and can replace technicians[27]. Moreover, most laboratories deal with number of plant species with complex characteristics and tissue culture protocols. It is highly unlikely that one type of robot can perform tasks for all the species. Maintenance of robots are also complex and require skilled personnel (the cost of such a person would be much higher than a tissue-culture technician).

Development of automated systems

A number of automated systems based on liquid media have been developed. Circulating liquid medium capable of being continuously or intermittently pumped eliminates the need to subculture plants due to depletion of nutrients[28]. Purging of some contamination and metabolites along with pH control can be performed automatically.

Harris and Mason[29] constructed two machines for agitation of plant cultures in liquid medium. One machine provided variable tilt and vibration and the other provided rocking action for culture flasks. Cultured grape shoots resulted in 7-fold increase in a number of regenerated shoots as compared to explants maintained on agar medium.

Maene and Debergh[30] added liquid medium to established but exhausted cultures and were able to achieve increased biomass production. Aitken-Christie and Jones[31] followed a similar procedure with twice weekly addition of liquid medium to cultures growing on semi-solid medium, resulting in incresed numbers of shoot cultures of *Pinus radiata*.

Tisserat and Vandercook[28] developed a partially automated liquid type system based on large elevated culture chamber which was periodically drained and refilled with fresh liquid medium (Figure 1). The control of liquid flow was governed by a peristaltic pump which was monitored by a computer. Increased growth rate with reduced labour requirements were achieved using this system. Farrel[32] developed a system in which circulating liquid medium was passed through multiple vessels not unlike the vessel used by Tisserat and Vandercook[28]. Farrel[12] used a synthetic hydrophillic hollow fibre material similar to plant xylem fibres to wick liquid medium from a reservoir into a sealed propagation chamber.

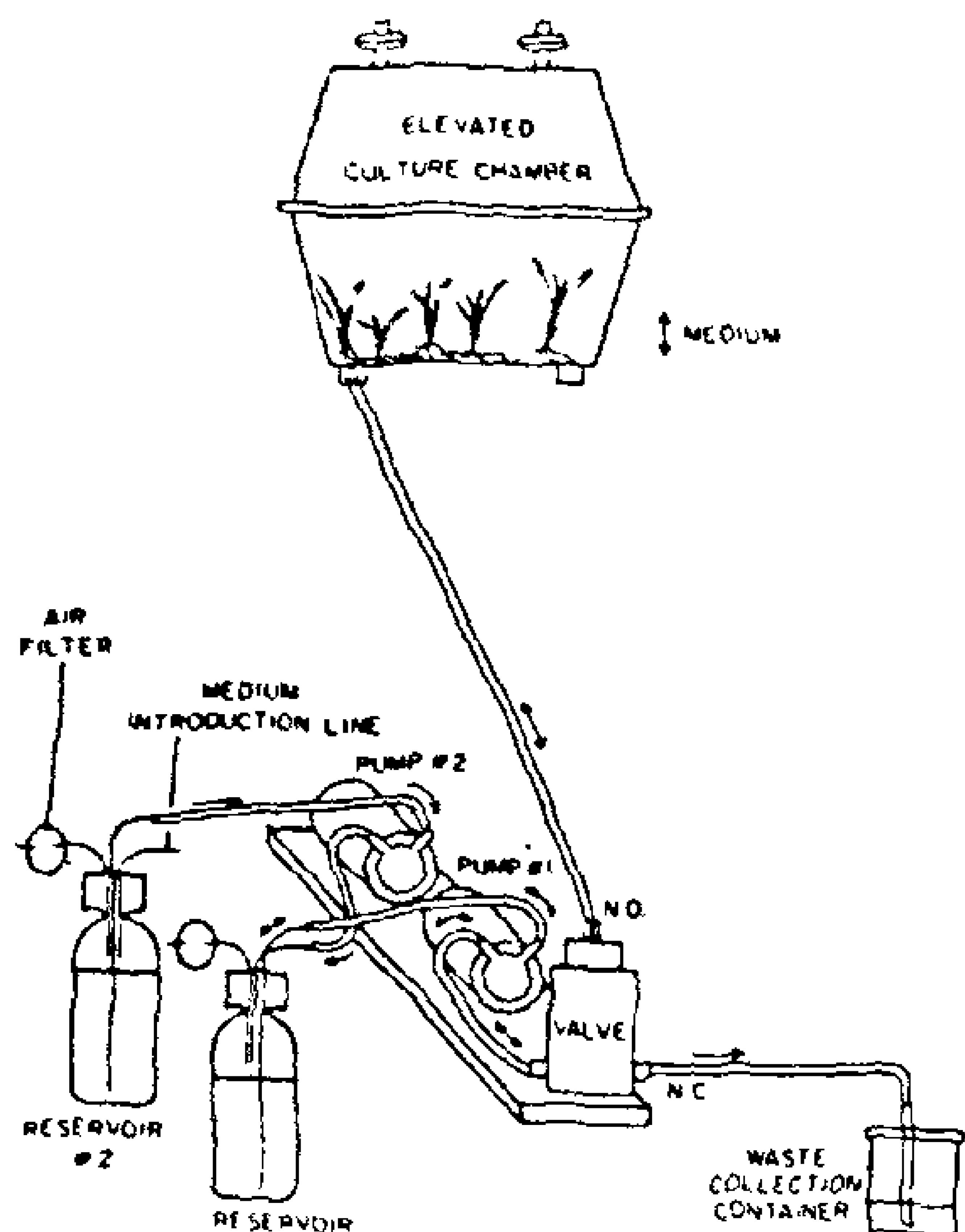

Figure 1. A partially automated system[28] based on elevated culture assembly. (Reprinted with permission from the publisher.)

Levin[33,34] and Levin *et al.*[19] described a process, based on liquid medium, and components for an automated plant tissue culture system. The system uses a homogenizer to separate densely growing meristematic tissues termed 'meristemoids'. Meristemoids are produced in liquid submerged conditions as short, miniature shoots with many axillary buds. It is possible to produce large numbers of meristemoids in limited volume of liquid. The meristemoids after being homogenized are passed through a sieving device to obtain small clusters of tissue of fairly uniform sizes and free of debris. The system also consists of a bulk tank to dilute sized tissues in sterile aqueous medium for dispersing into individual culture vessel for plantlet development. A microprocessor-controlled transplanting machine was incorporated for removing plantlets from the culture vessel to nursery trays.

The standard method of aerating liquid cultures by shaking or tumbling[28] may not be adequate for all plants and, therefore, the technique of aeroponics can be considered. In this technique nutrient medium is applied in the form of a fine mist. Weathers and Giles[35] developed a mist bioreactor in which plant tissues were grown on a biologically inert, fine mesh screen within a sterile chamber. The nutrient mist was sprayed from above on to the propagating tissue. Fox[36] also developed a similar mistifier although he generated the mist using a sonicator. Growth rates of 3.5 times greater were achieved using this technique than on agar culture. An ideal aeroponic unit to produce autotrophic cultures is shown in Figure 2.

Two main problems can be encountered when liquid medium is used, e.g. vitrification and contamination. Vitrification is the development of plants with 'glassy' appearance with nonfunctional stomata. Such plants also show decreased multiplication rates and are difficult to root[37]. Contamination, always a threat to cultures, can be more difficult to control in liquid medium. This is particularly important for the development of automated system where large numbers of plants are produced in a container. Under such conditions, if one plant gets contaminated the whole lot will have to be discarded. Because of these problems some commercial laboratories have reverted to semi-solid media[38].

There have been attempts to overcome these problems. Vitrification can be controlled by various additives in the medium, e.g. $AgNO_3$ or proper ventilation in the culture assembly or bottom cooling[30]. However, the problem of contamination is difficult to deal with. Sucrose-free medium has been considered by many workers to reduce the chances of contamination[39]. Sucrose-free medium offer additional advantages in generation of photoautotrophic cultures which are amenable to large-scale culture techniques. Fujiwara *et al.*[39] were the first to develop a system with minimum risks of contamination using sucrose-free medium. They termed the system 'photoautotrophic tissue culture system' (PTCS) which consists of a culture box assembly, a gas flow assembly and a culture solution flow assembly (Figure 3). Increased biomass yield was achieved when the assembly atmosphere was enriched with CO_2. In recent years, a number of plant species have been successfully cultured on sucrose-free medium, e.g. potato[40], carnation[41], strawberry[39] and roses[42]. This, therefore, provides the opportunity of developing tissue-culture protocols for automated systems.

Sharma *et al.*[43] developed a system (Figure 4) in

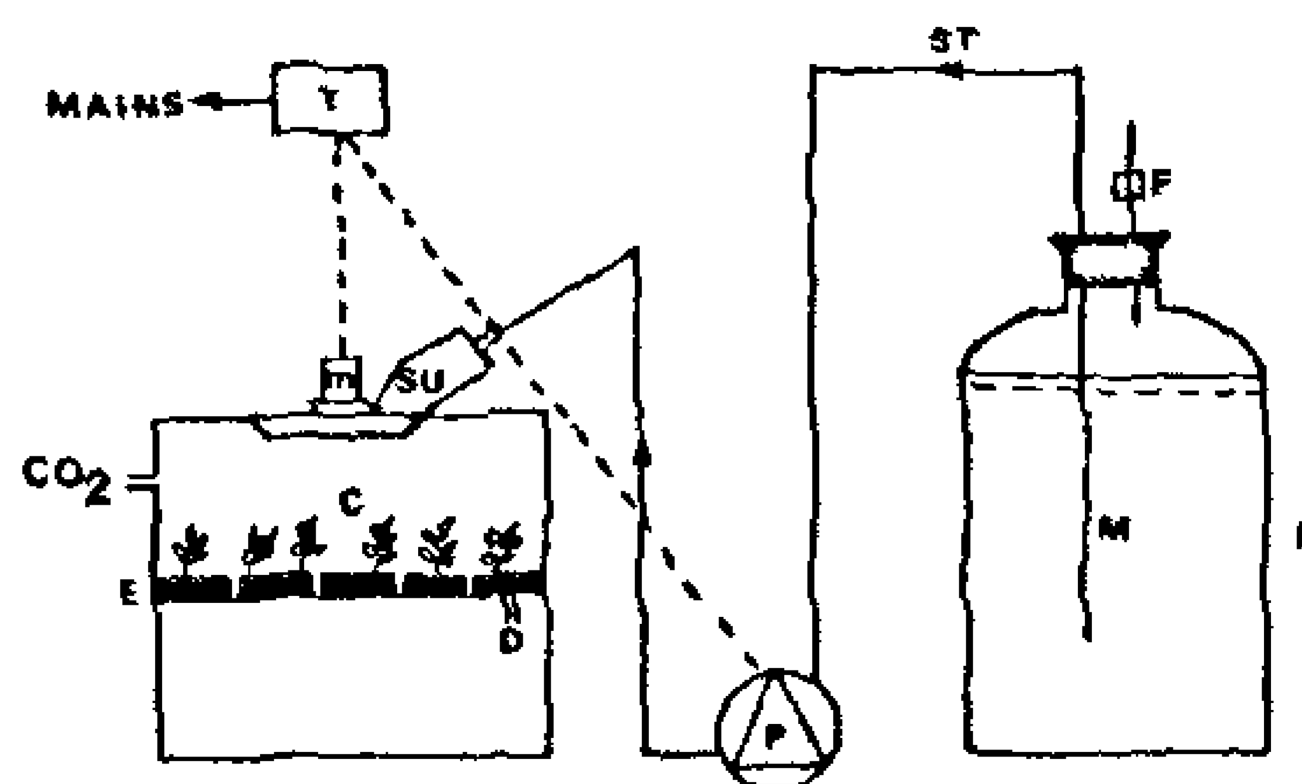

Figure 2. An aeroponic unit to produce autotrophic cultures. T, Timber unit; m, 9-V motor; SU, spraying unit; E, Elisa plates; C, transparent chamber, D, drainage; P, Peristaltic pump; ST, silicone tubing, F, air filter, M, medium; R, reservoir (N. K. Sharma, unpublished)

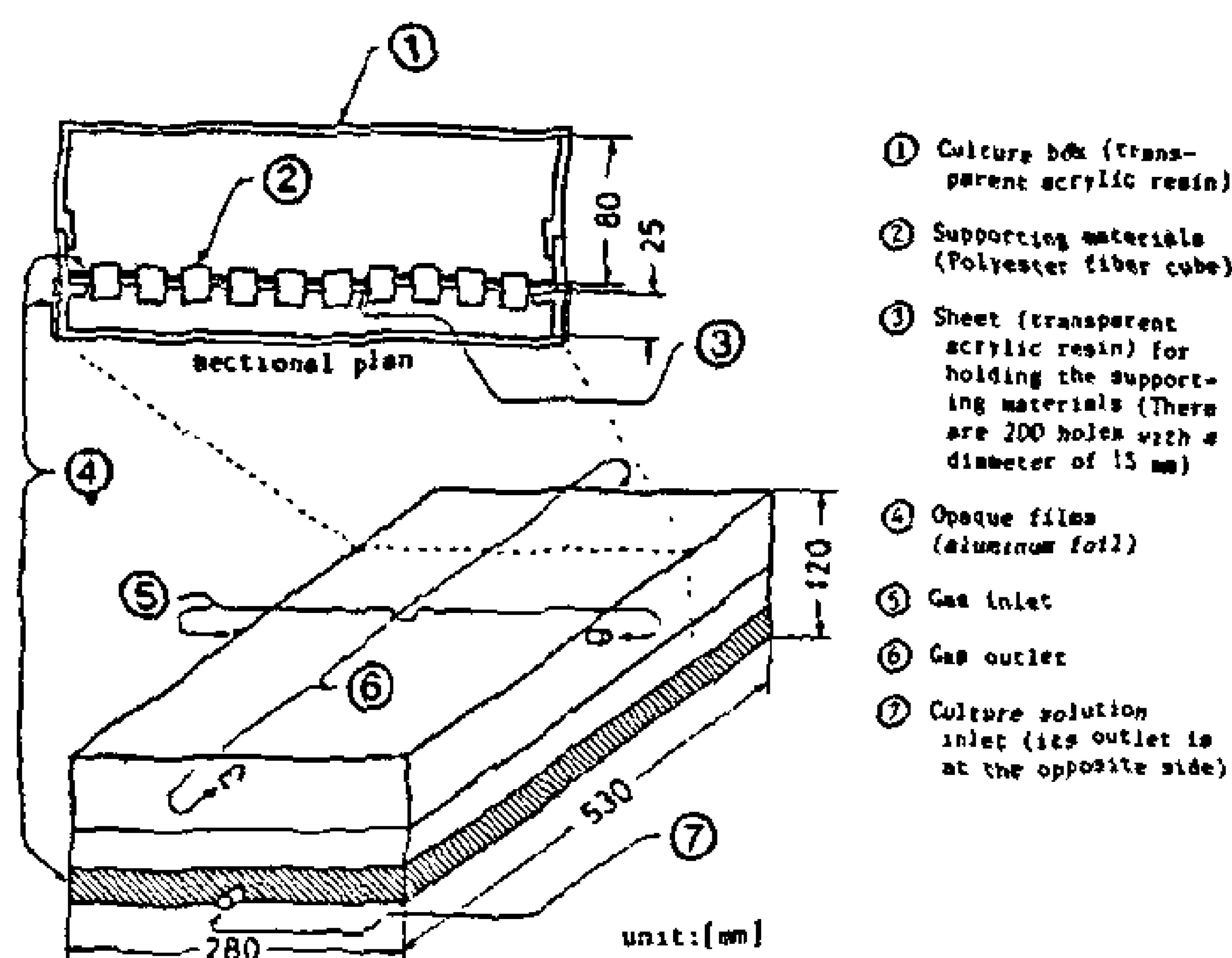

Figure 3. Photoautotrophic tissue culture system[39]. (Reprinted with permission from the editor.)

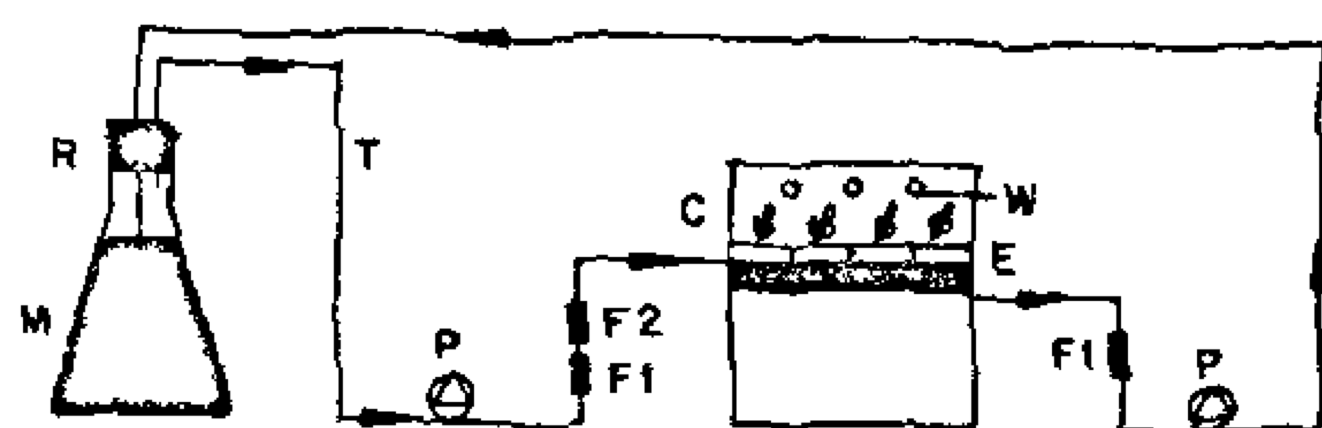

Figure 4. A system to acclimatize micropropagated plants[43]. M, *Medium; R, reservoir; T, tubing; P, peristaltic pump; F1*, 0.43-μM filter; F2, 0.22-μM filter; E, Elisa plates; W, 0.4-cm-wide windows.

which plants were cultured on circulating liquid medium without sucrose inside a transparent chamber on floating Elisa plates. The plates were drilled so that the medium comes in contact with the base of the plants. The cultured chrysanthemum plants showed improvements, compared to *in vitro* grown plants, with respect to increased leaf area, reduced stomatal index, reduced water losses from leaves and had increased survival when transferred to soil. It is suggested that the system could provide an useful way of acclimatizing the cultured plants, thus eliminating the need of having a mist chamber or glass house with such facilities.

Thus, tissue-culture technology is passing through an era of mechanization and automation and it will not be too long when large numbers of superior and disease-free plants species will be available. This will also encourage the growth of tissue-culture industry due to reduction in production cost. However, many problems remain to be solved, e.g. optimizing nutrient medium for different species/culture conditions for development of autotrophic tissue culture systems.

Conclusions

The use of plant tissue culture techniques has revolutionized the plant propagation. However, the current methods used for rapid clonal multiplication have several drawbacks that limit its production at large scale.

Somatic embryogenesis and liquid-based cultures have the potential for automated mass propagation systems. The bioreactor technique, with modification, can be employed for large-scale somatic embryogenesis. However, this technique can be adopted only for those species where the technique of somatic embryogenesis has been achieved.

The continual flow, liquid nutrient offers significant advantages over agar-based systems for enhanced growth and feasibility of mechanization of plant micropropagation. Several systems have been developed based on this technique. However, a significant cost reduction can be achieved using sucrose-free medium. The autotrophic plant culture systems have advantages over other systems as it produces autotrophic plants and also reduces the chances of contamination.

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