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APPROACHING MECHANIZATION OF PLANT MICROPROPAGATION

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ABSTRACT
Investigations of materials and methods for growing plant tissue in a continual-flow, liquid nutrient medium as an alternative to semisolid agar medium have been made. Enhanced growth of plant tissue on microporous polypropylene membranes floating on a liquid nutrient has been demonstrated. Moreover, in vitro plantlets on the microporous membrane are free from entanglement with the support matrix and readily available to mechanized handling. Trained growth of plantlets through polypropylene netting shows potential for mechanization by mass handling (separation, singulation, and transfer) of plant tissue cultures. Keywords: Plant tissue culture, Micropropagation, Mechanization.

OBJECTIVES
The objectives of this research were both short term and long term. In the short term, the objective was to develop a continually replenishing, liquid media culture system and to identify and test alternative materials, or matrices, for physically supporting plant tissue to accommodate aeration characteristic of conventional agar cultures. The long term objective of this research was to establish techniques and systems that will be compatible to mechanized handling, separation, singulation and transfer of plant micropropagation.

REVIEW OF LITERATURE
Bioreactor technology is quite sophisticated for microbial and mammalian cell culture and even plant cell culture, where the major product is a secondary metabolite. Fermentors, chemostats, and phytostats are common commercial bioreactors for both batch and continuous industrial cell culturing. With plant micropropagation, however, the desired final product is a whole plant. Commercial bioreactors are not generally compatible to this kind of end product, although they may provide needed features of aseptic and controlled environments.

Growth media for plant tissue culture may assume liquid (suspension) and solid (agar) forms, yet agar is most widely used both in laboratories and in commercial settings. Except for scale, commercial tissue culture facilities strongly resemble research laboratories in equipment and procedures. Propagation containers are numerous, individual, and small. They consist of test tubes, Petri dishes, flasks, and jars. Large numbers of small containers make labor intense, routine, tedious, and often dull, particularly on the commercial scale (Wochok, 1979).

Maene and Debergh (1985) tried to reduce manual labor by adding liquid media to established, exhausted agar cultures instead of transferring tissue to new vessels with fresh agar media. Through media modifications they were able to achieve elongation and enhanced rooting, but they encountered increased vitrification from submersion in the liquid. Subsequently, Maene and Debergh (1986) used bottom cooling of the culture medium to lower relative humidity of the container atmosphere in the liquid-over-agar vessels and, hence, to produce shoots without vitrification. Aitken-Christie and Jones (1987) applied twice weekly flushings of liquid medium over agar for periods of 4-6 h to produce radiata pine shoot hedges in vitro. Shoots of 20-30 mm lengths were selectively harvested with scissors each month for an 18-month period without transferring to fresh agar medium. Shoots retained on the initial agar media and flushed with liquid media had
elongation significantly greater than shoots conventionally transferred to fresh agar media. Aitken-Christie and Davies (1988) expanded their earlier jar cultures of shoot hedges to a large (390 mm long x 250 mm wide x 120 mm high), clear, autoclavable, polycarbonate container with automatic addition and removal of liquid nutrients controlled by peristaltic pumps and programmable time clocks. Problems with sterility were encountered under normal laboratory laminar flow bench conditions.

Tisserat and Vandercook (1985, 1986) constructed an automated plant culture system (APCS) which consisted of a two-piece polystyrene chamber (320 mm long x 175 mm wide x 165 mm high) to which periodic floodings of liquid nutrient were controlled by a computer. Explants of orchids, carrot, date palm, aster, and cow tree were positioned within compartments of plastic ice cube trays with holes drilled in their bottoms. Tissues were immersed by liquid nutrient flooding every 2 h. Nutrient was then allowed to gravity-flow back into the supply reservoir, leaving the plant tissue to rest on glass beads where it received aeration (Wood, 1985). The chamber was not autoclavable and had to be sterilized by 12% ethylene oxide gas for 2 h. Growth of plants either equaled or exceeded that of plants on concurrent agar media cultures. After nine months of culture in the APCS, orchid tips produced four times as much tissue as orchids transferred every six weeks to fresh agar media. Enhanced growth was attributed to replenished liquid nutrient and a larger, non-constraining vessel environment for aeration and release of toxins. Simonton and Robacker (1988) developed a liquid nutrient system which supplied several 10-L polycarbonate containers as separate culture vessels from a central supply carboy. They had limited success with a chemical sterilization scheme of detergent, alcohol and sterile water rinses between culture cycles to avert time-consuming and difficult autoclavings. Farrell (1987) reported that Agro-Clonics, Inc., Orange, CA. made an automated plant micropropagation system by the trademark of Mega-Yield. This system used liquid nutrient media flowing through multiple vessels similar to the vessels used by Tisserat and Vandercook (1985). Farrell used a synthetic hydrophilic hollow fiber material adapted from the biomedical industry to mimic the plant's xylem fibers to wick liquid nutrient media from a bottom reservoir into a hermetically sealed propagation chamber. Growth performance with the Mega-Yield system is not in the published literature.

Farrell (1988) described the Propamatic micropropagation system developed at the Research Institute for Vegetable Crops (ZKIFV) in Budapest, Hungary. The Propamatic system was manufactured by Kontakta, a Hungarian company. The system was essentially an automated laminar flow workstation that dispensed special, small, rectangular plastic propagation vessels (called Veg-Box) and mixed and injected aseptic liquid-state agar media with a device called Clonmatic.

Levin (1983, 1984) and Levin et al. (1988) described the process and components for an automated plant tissue culture system marketed by an Israeli company, P.B. Ind., under the identity of Vitromatic. The system was liquid nutrient based. The process used a homogenizer device (similar to the concept offered by Cooke, 1979, for ferns) to separate densely growing meristematic tissue. A sieving apparatus sorted small clusters of tissue of fairly uniform sizes and free of debris. Then a bulk tank allowed dilution of sized tissue in a sterile aqueous medium for dispensation into individual culture vessels for plantlet development. The separation (homogenizer) step was eliminated with embryogenic cultures. A microprocessor-controlled transplanting machine was reported for removing plantlets from the culture vessels and placing them into nursery trays at a rate of about 8,000 plantlets/h.

A nutrient mist bioreactor for plant tissue culture was developed by Weathers and Giles (1988). Tissue was grown on a biologically inert, fine mesh screen within a sterile chamber. A nutrient mist was sprayed from above onto the propagating tissue. Fox (1988) described a commercial version called the Mistifier being developed by BioRational Technologies (Stow, MA) and to be manufactured and marketed by Manostat (New York, NY). Growth rates of 3.5 times greater in the mist bioreactor than on conventional agar cultures were cited. It was also observed that callus in the mist bioreactor exhibited a bulbous, almost spherical growth pattern compared to rather erratic growth patterns on agar.

Hamilton et al. (1985) reported a technique for growing micropropagules on flat microporous polypropylene membranes floating on liquid nutrients. Sinking of the membrane as the weight of the plant material exceeded its buoyancy was a problem. Kong and Chin (1988) observed improved growth of asparagus protoplasts on microporous polypropylene membrane. They surmised that the technique allowed the cultures to receive both aeration and nutrients without agitation needed with suspension cultures. Matsumoto and Yamaguchi (1989) used several synthetic nonwoven materials as supporting agents for in vitro culture of banana protocorm-like bodies. For reuse and plant growth, a 50% acetate and 50% polyester fiber gave the best compromise performance, considerably superior to agar.

**METHODS AND EQUIPMENT**

**CONTINUAL-FLOW, LIQUID NUTRIENT VESSELS**

Unlike liquid suspension cultures where plant tissue is immersed in static or oscillating liquid in a sealed vessel, the concept of continual-flow periodically flushes the vessel with fresh liquid nutrient. The plant tissue does not have to be disturbed by transfer to another vessel to receive fresh nutrients. An initial continual-flow apparatus was constructed by plumbing 20 Magenta GA-7 containers together through orifices in their bottoms. An in-line, thin-walled polypropylene, bellows diaphragm pump transferred liquid from a supply media glass carboy through a manual three-way valve into the Magenta containers. When the desired level was attained, the pump was shut off. Spent media was gravity-drained through the third port of the valve to a separate waste carboy. The principal of this system was very similar to the Tisserat and Vandercook (1985) system. Various support materials were used in the Magenta containers for growth comparisons.

A second-generation continual-flow apparatus used modified, 7-in. diameter, heavy duty glass beakers with media supplied by siphoning or a peristaltic pump (fig. 1). The beakers were closed by flat glass tops seated on flat ground beaker rims. Liquid media entered each beaker.
Bioreactors
Supply Reservoir
Peristaltic Pump
Gas Sterilization Filters
Waste Reservoir

Figure 1—Schematic of continual-flow, liquid nutrient culture apparatus.

through a port in the sidewall of the beaker approximately 50 mm above the bottom. This port was sufficiently high above the bottom of the beaker to assure a physical discontinuity of liquid between the beaker and the supply lines to restrict contaminant transport. Spent media was drained at desired intervals by gravity flow through the bottoms of the beakers.

ALTERNATIVE SUPPORT MATRICES
The most apparent deficiency of liquid suspension culture compared to agar culture is its inability to support the tissue so that it receives sufficient aeration for non-vitrified growth. Consequently, as we initiated our work with continual-flow liquid culturing, we surveyed various materials as alternatives to agar for physical support of tissue. Based on criteria such as autoclavability, strength, absorbancy, inertness to plant tissue and reusability, we screened approximately 30 material options including paper, plastics and wire screening. Five materials were selected for testing: polyurethane foam, nonwoven polypropylene fiber, glass beads, microporous polypropylene membrane and polypropylene netting.

Each of the materials were used as growth support matrices in both static vessels and in continual-flow vessels. The static liquid medium tests were conducted in Magenta GA-7 containers, square plastic vessels designed specifically for the plant tissue culture industry. Continual-flow tests were conducted both in Magenta vessels and in large, modified glass beakers.

MEMBRANE/NETTING GROWTH DEVICES
The microporous polypropylene membrane (Celgard® membrane by Hoechst Celanese, Separation Products Division, Charlotte, NC) was a very intriguing support material which had compatibility with mechanization. It floated on liquid and would physically support limited biomass as a flat sheet in direct contact with the liquid medium. As the tissue grew, however, the membrane tended to sink. Several configurations of devices utilizing this membrane were tried to assure floating as the plant tissue expanded. One of the earliest configurations had the flat membrane sheet pinched and glued at the corners into “dog-ears” to curl the sides up. Another configuration had the membrane glued onto a square rim of closed cell polypropylene foam to enhance buoyancy. Hoechst Celanese also provided a limited number of membrane “boats”. These boats had a square solid polypropylene sidewall heat-sealed to the membrane which served as a bottom. Later this configuration was constructed using different adhesives to glue the membrane to the sidewall frame.

A second material, a polypropylene netting, was tested both as an alternative support to agar and as a device through which to train growth of plantlets. An 8x8 mesh (i.e., 8 grids/in, in a square pattern) of this netting in a 50 mm square, trifold configuration was initially used with explants placed between the bottom and middle layers. As the explants callused and produced shoots, they grew through the top two layers. Separation of the plantlets from the callus and regenerating tissue was attempted by two actions: 1) peeling the top layer away from the middle layer; and 2) shearing the top layer laterally past the middle layer. The trifold netting was used in the continual-flow, liquid nutrient vessels and Magenta vessels both on top of other support materials and alone.

More recently, devices that combined the two materials, microporous membrane and netting were tested. Initially, a second rigid frame was wedged inside the sidewalls of a membrane boat to tighten the netting at 5-10 mm above the membrane (fig. 2). In a second “sandwich” device, the netting was attached to the top edge of the solid sidewall frame and the membrane was attached to the bottom edge as shown in figure 3. In both devices, the explant was dropped through the grid of the netting for culture initiation. Plantlets grew up through the netting.

PLANT MATERIAL AND MEDIA PREPARATION
Tomato (Lycopersicon esculentum cv. Rutgers) seeds were disinfected by soaking in 10% Clorox for 10 min and rinsed twice in sterile, distilled water. Seeds, 15-20/plate, were then placed in sterile glass Petri plates containing filter paper and water. The plates were incubated at 27° C with a 16 h daylength under fluorescent lights. Cotyledons were aseptically removed after 7-10 days germination and planted in vitro in Magenta GA-7 vessels and the continual-flow beaker system. Fresh weights were recorded as grams/vessel. Culture media used was Murashige and Skoog salts (Murashige and Skoog, 1962) with 30 g/L sucrose and 2 mg/L zeatin. Growth data were recorded after 12-21 days as fresh weight gain. No dry weights were recorded.

Figure 2—Two-frame membrane-netting device.
Subcultures of begonia (*Begonia rex*) were made with small callus clusters (0.2 to 0.7 g fresh weight) on Murashige and Skoog salts containing 30 g/L sucrose, 0.17 g/L NaH₂PO₄, 0.08 g adenine sulfate, 0.1 g/L i-inositol, 0.0004 g/L thiamine HCl, 10 μM indole acetic acid and 10 μM kinetin at a pH of 5.7. Seven grams per liter of agar were used in agar treatments with 40 mL of that medium added to each Magenta GA-7 vessel. Forty milliliters of liquid (agarless) medium was used with Celgard® microporous membrane rafts (Sigma Chemical Co., St. Louis, MO). Thirty milliliters of liquid medium was used in Magenta GA-7 vessels containing 5x5x1 cm squares of nonwoven polypropylene fiber treated with Decerisol (a non-toxic surfactant). Thirty milliliters of liquid medium was also used with 3 mm diameter glass beads at a height of 3 cm in the Magenta vessel. Growth data were recorded after 25-33 days as final fresh weight. Dry weights were determined after 24 h of drying at 65°C (140°F).

All Magenta GA-7 vessels were autoclaved with the solid matrices and medium in place. For the continual-flow (beaker) vessels, 3 to 4 L of media were autoclaved in 9-L glass carboys for 1 h at 121°C and 18 psi pressure. Sucrose and zeatin were filter sterilized in a 150 mL volume through a 500 mL, 0.2, μM SuperVac vacuum filtration unit (Gelman 3010). This solution was then quickly added to the supply carboy under the laminar flow hood and mixed. The overriding principle of this procedure was to transfer minimum volumes of medium for a minimum length of time with the smallest vessel openings possible. It is best to complete the medium sterilization two days prior to culture initiation to monitor for contamination. All remaining system components were individually wrapped in brown, lint-free paper and steam sterilized. The paper was removed only under the laminar flow hood when ready to use.

RESULTS AND DISCUSSION

CONTINUAL-FLOW, LIQUID NUTRIENT SYSTEMS

The initial continual-flow, liquid nutrient system using Magenta vessels had several serious flaws. Crossover of fresh and spent media through common tubes between the three-way valve and the Magenta vessels encouraged rapid spread of contamination. The varying coefficients of expansion and contraction of the different materials used to construct the vessels, fittings, valves and tubing caused increasingly severe leakage (and perhaps contamination) problems after each subsequent autoclaving. Moreover, the thickness of the polypropylene bellows diaphragm pumps was too small to withstand autoclaving without permanent deformation. This system was too complicated and not dependable.

The beaker vessels have proven to be superior to the Magenta system. The larger volume of the individual vessel reduces the number of connections (potential leaks). Positioning of the supply port 50 mm above the bottom of the beaker creates a physical barrier to rapid migration of contamination back to the supply medium. Either the siphon or the peristaltic pump eliminates the sterilization difficulties associated with an in-line, diaphragm pump and is simpler. Separate inflow and outflow media lines eliminated crossover contamination. Even while working under a laminar flow hood, one must exercise extreme caution to open the large top of the beaker only partially when inserting plant tissue. In the future, this or a third generation vessel will be instrumented for monitoring parameters of the medium and the atmosphere to establish criteria for environmental control of the continual-flow concept.

SUPPORT MATRICES

Early in the growth performance testing of polyurethane foam, it became evident that the wicking action was too slow. Tomato cotyledons would desiccate before liquid nutrient became available to them. Nonwoven polypropylene fiber wicked liquid more readily than polyurethane foam, but lacked uniformity of wetting at the surface. Explants frequently desiccated on this material also. A non-toxic surfactant (Decerisol) increased the rate of wicking and improved uniformity of wetting. Mixing and maintaining sterility of the surfactant, however, were additional complications. Intertwining of regenerative tissue in the nonwoven fiber was observed. This condition discouraged use in a mechanized separation operation.

Glass beads provided adequate support of micropropagules, but were very sensitive to maintaining a liquid level in contact with the bottom of the plant material to avert nonoptimal growth or desiccation. Tisserat and Vandercook (1985), no doubt, found a need for frequent cycles of immersion and draining of media in their APCS system. Their mode of liquid replenishment probably required greater volumes of nutrient medium than infrequent floodings of liquid around the beads to a level contacting the bottom of the plant tissue. A precise level of liquid medium, however, was not readily achieved and maintained. Perhaps the greatest detriment to the use of glass beads in a mechanical system is the inability of loose beads to maintain shape integrity. They must be restrained by a carrier apparatus to which they can conform in shape.

The trifold polypropylene netting functioned similar to glass beads, although it did provide a defined form. Liquid nutrient had to be recycled with intervening periods of draining to avoid vitrification. When used on top of the other alternative support materials tested, trifold netting held the plant tissue too far from the upper surface of the wetting front and resulted in tissue desiccation. Other difficulties encountered with the trifold netting were achieving a flat conformation of the three folds and inserting explants and preventing their falling through the
open edges of the device.

The microporous polypropylene membrane provided physical separation of the tissue from the liquid nutrient yet allowed transfer of nutrient to the plant tissue through the micropores (0.075 μ). Its buoyancy enabled it to float a small biomass, even as a flat sheet lying on the liquid surface. It also provided essentially 100% surface contact with the liquid media, a feature which improved uniformity of tissue growth. However, additional buoyancy and/or edge walls were needed to prevent flooding as the tissue mass increased.

Figure 4 compares mean fresh weight gains for initiating cultures of tomato cotyledons and transfers of cultures of begonia in static medium in Magenta GA-7 vessels on four support materials: agar, Celgard® microporous membrane, nonwoven polypropylene fiber and glass beads. Four or five replications of each support matrix were made in Magenta vessels containing 4-6 tomato cotyledons of equivalent initial age, and combined fresh weights were recorded. Cotyledons on microporous membranes had the highest mean fresh weight gain of 0.77 g, significantly greater (α=0.05) than tissue grown on traditional agar (0.47 g). In fact, growth on microporous membrane was also significantly greater than growth on glass beads (0.26 g) and nonwoven polypropylene fiber (0.37 g). Fresh weight gain on glass beads (0.26 g) was significantly less than gain on agar.

Low growth performance on glass beads was probably a result of using a static liquid level in the Magenta vessels, unlike the periodic liquid medium flushings used by Tisserat and Vandercook (1985, 1986). Detrimental effects were observed of both too high or too low liquid medium levels within the culture vessel. Vitrification and rapid tissue demise resulted from too high medium, and desiccation resulted from too low medium. Medium levels in contact with the lower surface of the tissue were extremely critical on glass beads. Although not quite as critical, tomato cotyledons were similarly sensitive to medium level on nonwoven polypropylene fiber.

Figure 4 also shows mean fresh weight gains measured for transferred cultures of callus clusters of begonia tissue. Three replications of each of the four support matrices were used in Magenta vessels with static medium. Five to six clusters of begonia tissue divided from established in vitro agar cultures were transferred to each Magenta vessel. Combined weights of clusters in each vessel were recorded. Mean fresh weight gains of 7.60 g on microporous membrane and 7.64 g on nonwoven polypropylene fiber were essentially equivalent, and the highest for the four support matrices. A mean fresh weight gain of 4.08 g on agar was the lowest of the matrices tested. Glass beads yielded a weight gain of 6.46 g, which was not significantly different (α=0.05) from microporous membrane and nonwoven fiber. All three alternative matrices—microporous membrane, nonwoven fiber and glass beads—yielded significantly higher mean fresh weight gains of transferred begonia tissue than agar. Closer relative growth performance of begonia than tomato on glass beads, nonwoven polypropylene fiber and microporous membrane may have resulted because of greater care to maintain critical liquid levels, knowledge gained from the earlier tomato cotyledon studies. Moreover, the begonia tissue transferred from established in vitro (agar) cultures appeared to be less susceptible to contamination and perhaps more resilient to environmental shock than the initiation cultures of tomato cotyledons.

MECHANICAL SEPARATION

The trifold netting enabled examination of two methods of mechanically separating the plantlets from the calli and poorly differentiated tissue. When the upper layer of netting was lifted vertically, or peeled away from the middle layer, plantlets did not separate cleanly from the calli, but tended to strip through the grids of the netting and become damaged. On the other hand, separation of the plantlet from the calli was easily achieved by shearing the upper netting layer laterally past the middle layer.

The membrane-netting devices shown in figures 2 and 3 have enabled separation of plantlets from regenerating tissue by cutting along the upper or lower surface of the netting, as appropriate. The cut plantlets may subsequently be treated as unrooted cuttings and potentially transferred to nursery media after receiving rooting hormone treatment. Figure 5 shows a culture of sweet potato in the 1-piece “sandwich” device shown in figure 3. The “sandwich” device might become sufficiently cost effective to be disposable after 2-5 mechanical harvests of plantlets and the regenerative tissue has aged.

![Figure 4-Comparison of mean tissue fresh weight gains on four alternative support matrices.](image1)

![Figure 5-Sweet potato culture in the sandwich device.](image2)
SUMMARY AND CONCLUSIONS

The continual-flow, liquid nutrient concept offered advantages over agar and conventional suspension cultures for both enhanced growth and potential mechanization of plant micropropagation. The microporous polypropylene membrane achieved the aeration afforded by agar media yet allowed nutrient replenishment afforded by continual-flow liquid media. The trained growth of plantlets through the polypropylene netting could provide a tool for mechanized mass handling of transfer, separation and singulation functions in plant tissue culture. Mechanized handling of plant tissue should be planned to enable interface compatibility with greenhouse and/or field handling of the crop.

REFERENCES


